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Enrichment, isolation, and characterization of a nitropropanol metabolizing bacterium from the rumen

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Enrichment, isolation, and characterization
of a nitropropanol metabolizing
bacterium from the rumen

by

Robin Carl Anderson

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Departments: Microbiology, Immunology and Preventive
Medicine
Animal Science
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For the Graduate College

Iowa State University
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1995

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To Carrie, Nathan, Charles, and Austin

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CHAPTER 1. GENERAL INTRODUCTION

Ether glycosides of 3-nitro-1-propanol and glucose esters of 3-nitro-1-propionic acid occur in many legumes distributed throughout the world (40). In North America, more than 250 species and varieties of *Astragalus* are known to contain conjugates of either nitropropanol or nitropropionic acid (61-63). When these forages are consumed by ruminants, microbial enzymes within the rumen rapidly hydrolyze the conjugates to liberate the toxins, nitropropanol and nitropropionic acid (40). The structures of these toxins are shown in Figure 1. Reliable estimates of the number of livestock poisoned in the United States are not available, but estimates indicate that up to 5% of range cattle in British Columbia are poisoned each year by plants containing the nitro-toxins (33).

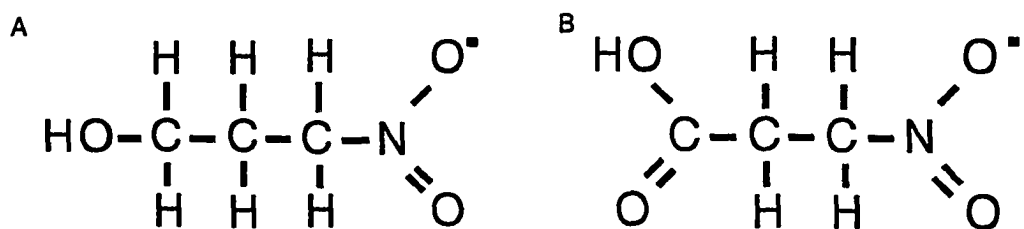


Figure 1. Chemical structures of 3-nitro-1-propanol (A) and 3-nitro-1-propionic acid (B).

Systemically, nitropropionic acid irreversibly inactivates succinate dehydrogenase (4, 16). Nitropropanol is not toxic per se, but is converted by hepatic alcohol dehydrogenase to nitropropionic acid (5, 9).

There is considerable evidence that ruminal microbes metabolize or detoxify the nitro-toxins (19, 37, 32, 33). Nitropropionic acid is metabolized more rapidly than nitropropanol (37) and in many instances plants containing the nitro-acid are safely utilized by ruminants (10-12, 19). At least 19 pure strains of ruminal bacteria are known to metabolize the nitro-toxins; however, at rates much slower than by mixed ruminal populations (32, 33). Thus, the contribution of these strains to detoxification is unknown. Rates of ruminal nitropropanol metabolism can be enhanced by modifying the rumen environment through dietary manipulations and this suggests that selection of nitropropanol metabolizing bacteria occurs (13, 31, 35). Possibly, the nitropropanol metabolizing bacteria selected are the major contributors to detoxification of the nitro-toxins.

This study was undertaken to define conditions which promote selection (enrichment) of nitropropanol metabolizing bacteria within mixed populations and to isolate and characterize nitropropanol metabolizing bacteria from these enrichments. This research is expected to expand the current base of knowledge regarding nitro-toxin metabolism and

information gained may enable the development of practical methods for prevention of nitro-toxin poisoning.

Dissertation Organization

This dissertation has been prepared in a non-traditional format. Chapter 1 includes a general introduction and review of the literature. Chapters 2 and 3 are manuscripts prepared for submission to Applied and Environmental Microbiology. Chapter 4 is a general summary and discussion. The literature cited in the introduction, literature review, and summary and discussion sections will follow chapter 4. Results from preliminary experiments on the biochemical reduction of nitropropanol are presented in an appendix section which appears at the end of the dissertation. The dissertation conforms to the style required by Applied and Environmental Microbiology.

Literature Review

Toxicity

Marsh and Clawson (45) first reported livestock poisonings caused by plants containing the nitro-toxins. Since that time, poisonings of cattle, sheep, goats, and horses by plants containing nitropropanol or nitropropionic acid have been documented (23). Stermitz et al. (58) were the first to identify the most common glycoside of

nitropropanol, miserotoxin (3-nitro-1-propyl- β -D-glucopyranoside) and several glucose esters of nitropropionic acid have subsequently been isolated (18, 40). Recently, intoxication of humans who consumed sugar cane contaminated with a nitropropionic acid synthesizing *Arthrrium* has been reported (30).

In ruminants, the glucose conjugates of nitropropanol and nitropropionic acid are rapidly hydrolyzed within the rumen (by microbial β -glucosidase and esterase, respectively) to yield glucose and free nitropropanol or nitropropionic acid (19, 70). Following hydrolysis, nitropropanol and nitropropionic acid are either further metabolized by the ruminal microbes or absorbed (40). Absorption of the nitrotoxins occurs primarily in the reticulo-rumen (42, 54). In nonruminants, esters of nitropropionic acid are hydrolyzed by mammalian esterase activity in the upper gastrointestinal tract; however, rapid absorption of the nitro-acid precludes its metabolism by microbes in the lower gut (40). In contrast to ruminants, nonruminants lack β -glucosidase activity in the stomach and intact miserotoxin is rapidly absorbed in the upper gastrointestinal tract. Little of the glycoside reaches microbial β -glucosidase activity in the lower gastrointestinal tract (41).

The absorbed glycoside is relatively innocuous to rats as it is excreted in the urine (41). Nonruminant animals are

therefore less susceptible to nitro-toxin poisoning by miserotoxin than to free nitropropanol (40). In rats for instance, the LD50 of miserotoxin is 2500 mg/kg body weight whereas the LD50 of nitropropanol is 77 mg/kg body weight (41). Rabbits are susceptible to poisoning by miserotoxin; however, evidence shows that the poisoning was due to methemoglobinemia rather than inhibition of succinate dehydrogenase (40). The methemoglobinemia resulted from the liberation of nitrite within the gastrointestinal tract without hydrolysis of the glycoside.

Various animal species have been used to elucidate the toxicity of the nitrocompounds and poisoning has been experimentally demonstrated in chickens, meadow voles, mice, pigeons, pigs, rabbits and rats (40). Following absorption, nitropropionic acid causes poisoning by irreversibly inactivating succinate dehydrogenase (4, 16, 21, 53). Absorbed nitropropanol is rapidly and irreversibly converted to the nitro-acid by hepatic alcohol dehydrogenase (5, 9, 42, 49, 50). Coles et al. (16) proposed that inactivation of succinate dehydrogenase results from the oxidation of nitropropionic acid to 3-nitroacrylate, which then binds to the enzyme. Alternatively, Alston et al. (4) proposed that the nitronate ion of nitropropionic acid irreversibly binds to the flavin component of succinate dehydrogenase.

There is no known antidote to nitro-toxin poisoning.

Inhibitors of alcohol dehydrogenase, such as ethanol or 4-methylpyrazole, slowed the transformation of nitropropanol to nitropropionic acid (40) but unless these inhibitors were given before the nitropropanol dose, poisoning was not prevented. A report that intramuscular administration of thiamine hydrochloride alleviated symptoms of nitro-toxin poisoning (51) has been challenged (40, 55).

Animals poisoned by the nitro-toxins exhibit a variety of symptoms depending on the severity of poisoning. Acutely poisoned animals may die within 4 to 25 hours (24). Less severely poisoned animals may show difficulty in breathing, muscular incoordination, depression, weight loss, and an increased heart rate (23, 24, 39, 43, 47, 48, 67, 69, 71). With cattle, a characteristic knuckling of the fetlocks has led to the term cracker heels because the heels often click together when the animals run (23). Frequent urinations and frothiness at the nose have also been observed (66). Characteristic with poisoned rats and meadow voles is an arching of the back (47). Chronically poisoned animals may remain affected for up to six months and may die at any time within that period if stressed (23).

Nitro-toxins in plants

In North America, nitro-toxin poisonings of ruminants occur most often in the Rocky Mountain regions (23) and the

milkvetchs *Astragalus miser* var. *oblongifolious* (Ryd.) Cron., *Astragalus miser* var. *hylophilus* (Rydb.) Barneby, and *Astragalus miser* var. *serotinus* (Gray) Barneby, have been implicated most often. However, plants containing nitropropanol or nitropropionic acid are distributed throughout the world. More than 450 species and varieties of *Astragalus* (Leguminosae) are known to contain either nitrotoxin, with over half of these plants present in North America (60-64). Other legumes known to contain nitropropionic acid are *Coronilla*, *Indigofera*, and *Lotus* (40). Nitropropionic acid is also synthesized by certain members of the Malpighiaceae, Corynocarpaceae, and Violaceae families as well as by certain species of fungi belonging to *Arthrinium*, *Aspergillus*, and *Penicillium* (19, 40).

Nitropropanol concentrations in *Astragalus miser* var. *serotinus* can exceed 4% of the plants dry matter (36) and, at a minimum lethal dose of 20 to 60 mg nitropropanol/kg body weight, a 500 kg animal can be poisoned by eating 0.6 to 1.8 kg of the plant. It is known that ruminants are more susceptible to poisoning by nitropropanol than nitropropionic acid; however, some plants that contain nitropropionic acid, such as *Astragalus canadensis* var. *brevidens* (Gand.) Barneby and *Astragalus falcatus* Lam., contain more than twice the amount of toxin found in plants that contain nitropropanol (23). Thus, on a dry weight basis, plants containing

nitropropanol or nitropropionic acid may be equally toxic.

Several factors contributing to increased accumulations of nitro-toxin within plants have been identified. Plants grown under conditions of restricted sunlight or with suboptimal moisture had reduced miserotoxin levels (39). Likewise, Parker and Williams (52) demonstrated that plants grown at 24°C had decreased levels of miserotoxin compared to plants grown at 32°C. They also showed that disrupting photosynthesis with (2, 4, 5-trichlorophenoxy)acetic acid or 2-(2, 4, 5-trichlorophenoxy)propionic acid resulted in decreased levels of miserotoxin. Miserotoxin levels in *A. miser* var. *serotinus* were unaffected by fertilization with ammonium nitrate, potassium nitrate, or ammonium sulfate, each at 56 and 112 kg nitrogen per hectare (52). In another study, Majak and Wikeem (44) found that fertilization with 100 or 200 kg urea nitrogen per hectare did not result in increased levels of miserotoxin during the first growing season. In plants grown with the 200 kg urea application, miserotoxin levels were increased during the second growing season but the authors could not rule out that the increased miserotoxin levels resulted from contributing factors such as reduced interspecific competition, residual nitrogen, or favorable moisture conditions. Levels of miserotoxin are highest prior to flowering and decrease as the plant matures (23, 38). Thus, to minimize the risk of poisoning,

management strategies rely on restricting the access of livestock to milkvetch range during periods of high miserotoxin concentration (69).

Ruminal metabolism and potential for detoxification

Microbes within the rumen are known to metabolize the nitro-toxins (19, 37), however, the rate of nitropropanol metabolism is much slower than that of nitropropionic acid (37). When given intravenously, nitropropanol is lethal to cattle at a dose of 30 mg/kg body weight (43); and nitropropionic acid is lethal to sheep at 52 mg/kg body weight (36). When given orally, the lethal doses of nitropropanol to cattle and sheep are 57 and 118 mg/kg body weight, respectively (18, 67); but because of microbial detoxification, the amount of nitropropionic acid required to cause poisoning is increased > four-fold (65, 68).

Majak and Cheng (32, 33) identified several organisms capable of metabolizing nitropropanol or nitropropionic acid. They found that 19 of 63 pure cultures of ruminal microbes metabolized nitropropanol or nitropropionic acid. Those capable of metabolizing the nitro-toxins were *Prevotella* (*Bacteroides*) *ruminicola*, *Desulfovibrio desulfuricans*, *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Veillonella alcalescens* and species of *Clostridium*, *Coprococcus*, *Lactobacillus*, *Ramibacterium* and *Peptostreptococcus*. Because

all of these organisms also reduced nitrite and because nitrite was found to accumulate in resting cell suspensions of *M. elsdenii* and of mixed ruminal populations, it was proposed that the nitro-toxins were cleaved to produce nitrite and the alkane. Detoxification was thus proposed to depend upon the subsequent reduction of nitrite. We recently found that this proposal was not correct since nitropropanol and nitropropionic acid are reduced primarily to their respective amines, aminopropanol and β -alanine, by mixed populations of ruminal microbes, although nitrite may be formed as a minor product (6).

Cattle diets can be manipulated to enhance rates of ruminal nitropropanol metabolism but the nature of this enhancement remains unclear. Animals grazing native ranges consisting primarily of Kentucky bluegrass, pinegrass, or bluebunch wheatgrass had microbial populations which metabolized nitropropanol more rapidly than cattle grazing alfalfa or orchardgrass (34). In this study, protein content of the forage was also shown to affect the rates at which microbial populations metabolize nitropropanol, with fresh orchardgrass pasture (25% crude protein on a dry matter basis) supporting higher rates than a ten-year-old stand of orchardgrass pasture (16% crude protein on a dry matter basis). In support of this, cattle receiving orchardgrass or timothy hay plus 0.5 kg soybean meal/head per day had

microbial populations that metabolized nitropropanol 40% faster than cattle not receiving the soybean meal supplement (31).

Evidence also suggests that nitropropanol metabolizing microbes can be selected from the mixed population. For instance, increased rates of nitropropanol metabolism have been associated with microbes collected from cattle receiving supplements of nitrate, sublethal amounts of *Astragalus miser*, or nitroethane, a less toxic analog of nitropropanol (13, 31, 35).

Rates of nitro-toxin metabolism by mixed populations of ruminal microbes are much faster than the rates measured in studies with any of a number of pure cultures (32). Also, since none of the pure cultures tested (32, 33) were isolated from populations with enhanced rates of nitropropanol metabolism, it is possible that the organisms primarily responsible for metabolism of the nitro-toxins have not yet been identified. Alternatively, suboptimal culture conditions may have been used.

Microbial nitrate metabolism

That rates of nitropropanol metabolism increased with nitrate supplementation (13) suggests that nitropropanol, nitrate and(or) nitrite may be analogous substrates for certain groups of bacteria. It has long been known that

nitrate is reduced within the rumen (29). Most of the nitrate is reduced to ammonia with very little nitrate metabolized by dinitrification processes (27). Aloboudi (1) found that *Clostridium* and *Peptostreptococcus* were the predominant nitrate-reducing bacteria in the rumens of unadapted and adapted animals. Other nitrate-reducing bacteria identified from the rumen were species of *Propionibacterium*, *Selenomonas*, *Bacteroides* and *Butyrivibrio*. Most of these nitrate-reducing bacteria also reduced nitrite (1). Cheng et al. (14) reported that 25 of 51 pure strains of ruminal bacteria in a culture collection metabolized nitrite.

There is considerable evidence that energy can be conserved during the reduction of nitrate and nitrite by anaerobic bacteria. For instance, strict anaerobes such as *Desulfovibrio* spp. and *Wolinella succinogenes* obtain energy by respiratory processes and are capable of using nitrate and nitrite as terminal electron acceptors (57; 72). Organisms such as *Clostridium perfringens* have been shown to conserve energy, as demonstrated by increased growth yields and fermentation balances, by using nitrate and(or) nitrite as electron sinks (20). Use of such electron sinks allow for more ATP production from substrate-level phosphorylation because NAD^+ can be regenerated without the diversion of acetyl-CoA to more reduced products such as butyrate.

However, not all anaerobes reduce nitrate to ammonia.

Several species of *Propionibacteria* reduce nitrate to nitrite and then to nitrous oxide (2, 26). Contrary to the reduction of nitrite by dinitrifying bacteria, energy is not conserved through this process by the *Propionibacterium* sp.. Rather, it has been postulated that this serves as a detoxification mechanism.

Considerable information exists regarding the processes and enzymes involved in dinitrification and in assimilatory nitrate reduction; however, much less information is available concerning nitrate reduction by ruminal microbes. One way in which assimilatory and dissimilatory processes have been distinguished is by the type of nitrate reductase (56). Assimilatory nitrate reductases (Pichonoty's Type B reductase) are soluble and are inhibited by chlorate. In contrast, dissimilatory nitrate reductases (Pichonoty's Type A Reductase) are membrane bound and chlorate is a substrate, although the reduced product, chlorite, is toxic to the cells. Assimilatory nitrate reduction is regulated by ammonia or other forms of reduced nitrogen and is relatively unaffected by normal atmospheric oxygen tensions (56). Dissimilatory nitrate reductases, in contrast, are unaffected by ammonia and are synthesized only under anoxic conditions. Dissimilatory nitrite reductases may or may not be membrane bound, depending on the species. Both assimilatory and

dissimilatory nitrate reductases contain haem and a molybdenum co-factor (59).

Anaerobic reduction of nitrate involves low potential electron carriers. Of the nitrate-reducing rumen bacteria, *Wolinella succinogenes* contains menaquinone and *b* and *c* type cytochromes (22, 28). Cytochromes of the *b* type were also found in *Selenomonas ruminantium*, *Anaerovibrio lipolytica*, and *Veillonella alcalescens* (17). Ferredoxin has been implicated as the electron carrier mediating the reduction of nitrate, nitrite and nitroethanol by *Clostridium* spp. (8, 15, 46). In this regard, it has been demonstrated that partially purified preparations of hydrogenase from *Clostridium pasteurianum*, when combined with ferredoxin, reduce nitrite as well as nitropropanol and nitropropionic acid (7). Rates of nitropropanol metabolism by the hydrogenase/ferredoxin system were stimulated by ferrous and sulfide ions. The stimulatory effect of these ions on the reduction of nitropropanol by mixed populations of ruminal microbes (6) supports the concept of a functional hydrogenase/ferredoxin system for the reduction of nitropropanol in the rumen.

Rapid rates of ruminal nitrate and nitrite reduction (3) require an abundant supply of reducing equivalents that arise from sufficient amounts of various fermentable energy sources. Lewis (29) demonstrated that H₂, formate, succinate, lactate, citrate, glucose, malate, or mannitol

were used by mixed ruminal populations to reduce nitrate. Possibly, the effect of the different diets on nitropropanol metabolism are likewise manifested by providing an abundant supply of reducing equivalents. Rates of nitropropanol metabolism were increased 24% in one experiment when molasses was supplemented to cattle grazing orchardgrass pasture; however, rates were not increased significantly in other experiments when cattle grazing alfalfa were supplemented with molasses or when cattle receiving alfalfa hay were supplemented with corn or silage (34, 35). *In vitro* experiments also suggest that providing a source of potential reducing equivalents, some which serve as donors for microbial reduction of nitrate, is not enough to stimulate nitropropanol metabolism (6).

Sheep have been successfully adapted to high nitrate diets (1, 3, 25) as have been cattle (13). Rates of nitrate and nitrite reduction increased rapidly during adaptation suggesting that this metabolic capacity was induced in the existing microbial population. However, proportions of nitrate and nitrite reducing organisms were also increased when high nitrate diets were fed (1, 3). Thus, the evidence suggests that certain species capable of reducing nitrate and(or) nitrite were enriched in the population.

The hypothesis underlying the research described in this thesis is that nitropropanol metabolizing bacteria can be

enriched by adapting populations to nitrate or nitropropanol. Isolation and characterization of the organisms that are most important in detoxification of nitropropanol would thus be facilitated from such an enrichment.

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**CHAPTER 2. ENRICHMENT AND ISOLATION OF A NITROPROPANOL
METABOLIZING BACTERIUM FROM THE RUMEN**

Prepared for submission to
Applied and Environmental Microbiology

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Abstract

Astragalus species (Leguminosae) containing the toxin nitropropanol are poisonous and cause considerable loss of productivity in livestock. Ruminants can acquire tolerance to nitropropanol when rates of ruminal metabolism are increased; however, the microbes responsible for the detoxification are not known. We report here the isolation of a ruminal bacterium able to metabolize nitropropanol. This isolation was from populations of bovine rumen bacteria that were enriched for nitropropanol metabolizing organisms during consecutive batch culture in medium containing nitropropanol. Our evidence showed that nitropropanol

metabolizing bacteria were present at approximately 10^4 organisms/ml in bovine ruminal fluid collected from a cow that had no prior exposure to nitropropanol and this number was increased 10,000-fold during enrichment whereas rates of nitropropanol metabolism were increased >8 fold. Our results indicated that phytone and H_2 appear to be substrates utilized as reductants by the nitropropanol metabolizing bacteria and either nitropropanol or nitrate can be used as electron acceptors.

Introduction

Nitropropanol and nitropropionic acid are toxic constituents of many forages consumed by ruminants. More than 450 species and varieties of *Astragalus* are known to contain either glucose esters of nitropropionic acid or ether glycosides of nitropropanol (17, 18, 19). Only one species has been found which contains both nitro-toxins (15). Esters of nitropropionic acid are also contained in certain species of *Coronilla*, *Indigofera*, and *Lotus* (13). These nitro-toxins are metabolized by ruminal microbes following hydrolysis from the glucose conjugates (6, 12). Nitropropanol is reduced to aminopropanol and nitropropionic acid to β -alanine as a result of microbial activity (2). However, ruminal microbes metabolize nitropropanol more slowly than nitropropionic acid and this difference contributes to the increased

susceptibility of ruminants to poisoning by nitropropanol (11, 12).

Majak and Cheng (8, 9) reported that of 63 pure cultures of ruminal bacteria tested, 19 were able to metabolize nitropropanol or nitropropionic acid. However, the rates at which these pure cultures metabolized the toxins were much slower than rates observed with mixed populations of ruminal microbes (8). This result suggests that suboptimal cultural conditions were used in these experiments or that the microbes predominantly responsible for metabolism of the nitro-toxins have yet to be determined.

Rates of nitropropanol metabolism vary depending upon animal diets (10) and are increased by supplementation of cattle diets with nitrate, protein, nitroethane, a less toxic analog of nitropropanol, or sublethal amounts of *Astragalus miser* (4, 7, 11). We believe that the latter observations suggest an enrichment of nitropropanol metabolizing microbes, and that such an enrichment provides the host with enhanced detoxification capabilities.

The objectives of this study were to enrich microbial populations for increased numbers of competent nitropropanol metabolizing microbes and to isolate such microbes from these enriched populations. Characterization of these isolates would then further our understanding of nitro-toxin metabolism in the rumen.

Materials and Methods

Sheep experiment. Three fistulated Border Leicester wethers (average weight 57.0 ± 11.4 kg) received a basal diet formulated to meet or exceed NRC requirements (14) and had ad libitum access to alfalfa hay. After 3 wk, the diets were modified such that one sheep received one of the following trial diets: basal plus 20 mg nitroethane/kg body weight per d (NE diet); basal plus increasing amounts of nitrate, 70, 110, 180, and 200 mg NaNO_3 /kg body weight per d on day 1 to day 3, day 4 to day 6, day 7 to day 9, and day 10 to day 28, respectively (NO diet); or basal plus substitution of orchardgrass with alfalfa (OG diet). The diets were fed in equal size meals (08:00 and 16:00) for 28 days. When supplied, nitroethane (sodium salt; 11), and nitrate were mixed with the concentrate portion of the diet. On days 28-37, the NE and OG diets were modified to contain 40 mg nitroethane/kg BW per d and to include 30 mg nitroethane/kg BW per d, respectively. The NO diet was terminated on day 28. The sheep showed no signs of toxicity from nitrate or nitroethane during the trial. Ruminal fluid was collected approximately two h after the morning feeding, strained through two layers of cheese cloth into prewarmed insulated containers and returned to the laboratory for *in vitro* determination of rates of nitropropanol and nitropropionic acid metabolism. Rates were determined 3, 2, and 1 days

prior to and at the indicated times after the start of feeding the trial diets.

Media and culture conditions. Medium A contained (mg/100 ml) K_2HPO_4 , 22.5; KH_2PO_4 , 22.5; $(NH_4)_2SO_4$, 45; NaCl, 45; $MgSO_4 \cdot 7H_2O$, 4.5; $CaCl_2$, 2.25; thiamine, 0.2; pantothenate, 0.2; nicotinamide, 0.2; pyridoxine-HCl, 0.2; riboflavin, 0.2; *p*-aminobenzoic acid, 0.01; biotin, 0.005; folic acid, 0.005; lipoic acid, 0.005; vitamin B-12, 0.002; resazurin, 0.1; cysteine-HCl, 50; Na_2CO_3 , 400; phytone peptone, 800; and clarified rumen fluid (40% vol/vol). Cysteine-HCl and Na_2CO_3 were added after the pH of the media containing all the other ingredients was adjusted to 6.8 with 30% NaOH. When used for roll tubes, medium A also contained 2% agar. Medium B was the same as medium A except it contained 8% instead of 40% clarified rumen fluid. In some experiments, where indicated, energy depleted rumen fluid (5) replaced clarified rumen fluid in medium B. Medium C contained, in addition to the ingredients in medium B; (200 mg each) cellobiose, glucose, soluble starch, xylose, trypticase, plus 8 ml milkvetch extract per 100 ml. Milkvetch extract was prepared by extracting milkvetch forage (ground to pass a 0.1 mm screen) with H_2O (16% wt/vol) for 2 h at 39°C with continuous agitation. Solids were removed from the extract by filtration. Media were dispensed under O_2 free gas into 4.5 ml or 9 ml volumes to 18 x 150 mm culture tubes, closed with

rubber stoppers and autoclaved in a press. Roll tubes contained 7 ml of medium.

In some tests, forages (milkvetch or alfalfa), ground to pass through a 0.1 mm screen, were added (0.1% wt/vol) to tubes prior to dispensing media. Additions of nitropropanol and other compounds were made by adding small volumes (<0.5 ml) of concentrated stock solutions. For cultures incubated with antibiotics, concentrated solutions of the antibiotics were dried under $H_2:CO_2$ prior to additions of forage and media. Kanamycin sulfate and chlortetracycline hydrochloride solutions were made in H_2O , all other antibiotic solutions were made in methanol.

Consecutive batch cultures were conducted essentially as described by Theodorou et al. (16) except that incubation times varied depending on the experiment. Initial cultures were inoculated (10% vol/vol) with rumen contents collected from fistulated cows maintained on an alfalfa:corn (9:1) diet. Ruminal fluid was collected approximately two h after the morning feeding, strained through two layers of cheesecloth into containers, which were capped, and returned to the laboratory for immediate use. After either 24 or 48 h of incubation, replicate tubes of each batch culture were combined (equal volumes) and used to inoculate (10% vol/vol) the next consecutive incubation series. This procedure was repeated every 24, or 48, h until the experiment was

terminated. When aliquots were withdrawn from cultures containing forage, pipettes with wide bore tips were used to allow particulate material to be transferred.

Most probable numbers (MPN) of bacteria were derived using a three tube method (3). Serial (10-fold) dilutions of mixed microbial populations (diluted in medium A) were inoculated into triplicate tubes containing medium A plus alfalfa or, for the secondary enriched population, tubes containing medium A supplemented with 4.2 mM nitropropanol. After incubation for at least 96 h, the samples were collected and analyzed for nitropropanol. Tubes were considered positive (at least 1 competent nitropropanol metabolizing bacterium was present in inoculum) if >75% of the added nitropropanol had been metabolized. Most probable numbers of total bacteria (those capable of growth in this medium) were estimated by scoring tubes (+ or -) for growth as determined by visual inspection of turbidity.

Anaerobic procedures were used in the preparation and inoculation of all media. Cultures were incubated at 39°C and, unless otherwise stated, the gas phase was H₂:CO₂ (1:1).

Determination of rates of nitropropanol metabolism.

Rates of nitropropanol and nitropropionic acid metabolism were determined from measurements of loss of nitropropanol or nitropropionic acid. Incubations were under CO₂ (sheep trial) or H₂:CO₂ (consecutive batch cultures).

The incubations were sampled while maintaining anaerobiosis and the samples were clarified by centrifugation (16,000 x g, 10 min) and stored at -25°C until analysis. Nitropropanol and nitropropionic acid were analyzed colorimetrically as previously described (2). Rates reported are those calculated during periods when the disappearance of nitropropanol or nitropropionic acid was linear. Typically, disappearance of either nitrocompound was linear from 0 to 8 h during incubations with concentrations of ≤ 8.4 mM but was linear over longer periods of time (>24) when concentrations were ≥ 16.8 mM.

Chemicals and reagents. Nitropropionic acid and nitropropanol were purchased from Aldrich (Milwaukee, Wis.) and Chem-Biochem Research, Inc. (Salt Lake City, Utah), respectively. Bacitracin was purchased from Calbiochem (La Jolla, Calif.). Other antibiotics were purchased from Sigma. Milkvetch forage, 4.4% miserotoxin content (7) was provided by Dr. Walter Majak (Agriculture Canada, Kamloops, B.C.). All other chemicals were of analytical grade.

Statistics. Unless indicated otherwise, rates reported here are the mean \pm SD from duplicate cultures. To test for increasing rates during the feeding trial, regression analysis was used to test for linearity. Rates of nitropropanol metabolism during consecutive batch culture were analyzed for differences using analysis of variance.

Results

Sheep trial. Ovine ruminal microbes metabolized nitropropanol and nitropropionic acid during *in vitro* incubation; however, the rates of metabolism varied considerably. Rates of metabolism by ruminal microbes were occasionally increased up to four-fold during the trial when sheep were fed the various trial diets; however, the lack of pattern for these increased rates suggest they reflect variability rather than actual changes in the microbial populations (Table 1). Since the rates did not increase linearly or markedly during the course of this feeding trial, and because of the large variability between measurements at different times, there was little evidence for selection of enriched populations of competent nitro-toxin metabolizing microbes. We thus decided not to attempt isolations from these populations.

Enrichment of competent nitropropanol metabolizing populations. Rates of nitropropanol metabolism increased during consecutive culture of mixed microbial populations in medium A plus added nitropropanol (Table 2). Similar increases (from <0.1 to >0.3 $\mu\text{mol/ml per h}$) were observed in other experiments when mixed populations were cultured in medium A plus milkvetch forage (which contains nitropropanol) but not when cultured in the medium A plus alfalfa but lacking nitropropanol. Most probable number estimates showed

Table 1. In vitro rates of nitropropanol (NPOH) and nitropropionic acid (NPA) metabolism by ruminal populations from sheep.

Days on trial diet	Diet ^a					
	Orchardgrass hay		Alfalfa plus nitroethane		Alfalfa plus nitrate	
	Rate of disappearance ($\mu\text{mol/ml/h}$)					
	NPOH	NPA	NPOH	NPA	NPOH	NPA
Pretrial ^b	0.13	0.22	0.03	0.13	0.06	0.06
1	0.28	0.24	0.08	0.13	0.08	0.02
7	0.07	0.17	0.10	0.13	0.04	0.10
13	0.08	0.11	0.03	0.06	0.04	0.03
20	NA ^c	0.0	0.0	0.07	0.0	0.04
29	0.11	0.12	0.10	0.10	NA	NA
36	0.0	0.01	0.19	0.41	NA	NA
Slope	-0.004	-0.005	-0.001	-0.002	-0.004	-0.001
R square	0.24	0.46	0.02	0.27	0.91	0.01
P-value ^d	0.51	0.21	0.83	0.37	0.05	0.96

^aDiets are described in materials and methods.

^bPretrial rates are the mean \pm SD of rates determined on three successive days immediately prior to feeding the treatment diets.

^cNA; not available.

^dTest for linearity (slope = 0).

that a population cultured two consecutive times in medium A plus alfalfa and 4.2 mM nitropropanol had increased concentrations of nitropropanol metabolizing bacteria (4.8×10^6 organisms/ml) compared to that in rumen fluid used as inoculum (3.0×10^4 organisms/ml). Deletion of phytone from medium A resulted in slower and smaller increases in rates of

Table 2. Mean rates of nitropropanol metabolism during consecutive batch culture of mixed microbial populations originating from two different cows.

Culture series	Rate of nitropropanol metabolism ($\mu\text{mol/ml per h}$) ^a	
	Population ^b	
	Medium A plus alfalfa and nitropropanol	Medium A plus nitropropanol
1st	0.05 \pm .01	0.01 \pm .01
2nd	0.43 \pm .04*	NM ^c
3rd	0.32 \pm .06*	0.04 \pm .05
4th	0.37 \pm .04*	0.07 \pm .01
5th	0.44 \pm .04*	0.17 \pm .06

^aValues are the mean \pm SD from two sets of serially transferred cultures; each set was inoculated with ruminal fluid collected from a different cow.

^bConsecutive batch cultures of the two mixed microbial populations were transferred at 24 h intervals. Nitropropanol addition was 4.2 mM to all cultures. Mean \pm SD rate of nitropropanol metabolism by microbes in ruminal fluid inocula was 0.11 \pm 0.02 $\mu\text{mol/ml per h}$.

^cNM; nitropropanol loss was not detected.

*Values within column are different than initial rate ($P < 0.05$).

nitropropanol metabolism. With 4.2 mM nitropropanol, elevated rates of nitropropanol metabolism were not maintained during continued transfer at 24 h intervals; however, elevated rates were maintained when twice as much nitropropanol was supplemented. Rates increased more rapidly and to a higher extent when alfalfa was included in

the medium (Table 2), although once enriched, the population could be maintained in medium lacking the forage component.

Rates of nitropropanol metabolism were always diminished when enriched populations were transferred into a medium containing a mixture of fermentable carbohydrates. For instance, when an enriched population was grown in medium C plus 4.2 mM nitropropanol, the rate of nitropropanol metabolism was less (0.07 ± 0.01 $\mu\text{mol/ml per h}$;) than that when the same population was inoculated into medium A plus milkvetch (0.29 ± 0.01 $\mu\text{mol/ml per h}$). Likewise, attempts to enrich populations were unsuccessful when the rumen fluid of medium A, supplemented with 4.2 mM nitropropanol, was replaced with 1% yeast extract and the forage component was substituted with any of the following substrates (10% wt/vol); arabinose, cellobiose, galactose, glucose, mannose, melezitose, raffinose, ribose, rhamnose, sucrose, trehalose, xylose, inulin, xylan, adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, fructose, or fucose.

Rates of nitropropanol metabolism were decreased 47% and 64% when the enriched population was incubated in medium B plus alfalfa, 4.2 mM nitropropanol and either 2 or 20 ug penicillin/ml, respectively, compared to the population cultured in the absence of added antibiotic (0.74 ± 0.04 $\mu\text{mol/ml per h}$). Similarly, rates of nitropropanol metabolism were reduced by 47%, 52%, 55%, 54%, 76%, and 65% when

populations were incubated with 20 ug/ml monensin, lasalocid, chlortetracycline, bacitracin, tylosin, or 40 ug/ml virginiamycin, respectively. Conversely, rates of nitropropanol metabolism were 0.82 ± 0.01 and 0.73 ± 0.05 $\mu\text{mol/ml}$ per h, respectively, for populations cultured in media containing 2 or 20 ug/ml of streptomycin.

Rates of nitropropanol metabolism in ruminal fluid from a cow, as well as in initial cultures (10% inoculum) in medium A plus milkvetch, were stimulated more than six-fold by 20 mM Fe^{2+} and 2.5 mM S^{2-} . However, populations possessing enhanced rates of nitropropanol metabolism as a result of consecutive transfer were not stimulated by Fe^{2+} and S^{2-} . Because 20 mM Fe^{2+} and 2.5 mM S^{2-} were likely to be inhibitory to growing cultures, rates in this experiment were determined by assaying aliquots withdrawn from 8 h old cultures.

We attempted to isolate nitropropanol metabolizing microorganisms from an enriched population established in medium lacking forage, where rates of nitropropanol metabolism averaged 0.48 ± 0.12 $\mu\text{mol/ml}$ per h after 5 consecutive 24 h incubations with 16.8 mM nitropropanol. The enriched population was serially diluted in medium A plus 33.6 mM nitropropanol and the dilutions were inoculated into roll tubes (in triplicate) containing the same medium plus 2% agar. After 48 h, total bacteria numbered 5.2×10^8 CFU/ml and 59 well-isolated colonies from tubes that had been

inoculated with 10^{-7} ml and 10^{-8} ml dilutions were picked to tubes containing medium B supplemented with 8.4 mM nitropropanol. None of these isolates metabolized nitropropanol during 168 h incubation. Nitropropanol was metabolized in tubes containing medium A plus 33.6 mM nitropropanol and inoculated with 10^{-5} to 10^{-8} ml of the culture that had been used to inoculate the roll tubes. Upon subsequent culture in medium A plus 33.6 mM nitropropanol, the population propagated from the original 10^{-8} ml inoculum metabolized nitropropanol at a rate of $0.49 \mu\text{mol/ml per h}$ ($n=1$). We have called cultures arising from this 10^{-8} ml inoculum our secondary enriched population. The concentration (MPN) of nitropropanol metabolizing bacteria present in this secondary enriched population (Table 3) was as high as 10^8 organisms/ml which was substantially higher than that in previous enrichments.

Development of media with greater selectivity for isolation of nitropropanol metabolizing bacteria. Studies were conducted with the secondary enriched population to develop media with greater selectivity for nitropropanol metabolizing bacteria. We found that while the rate of nitropropanol metabolism decreased little, the MPN of nitropropanol metabolizing microbes as well as the MPN of the total population were decreased when the secondary enriched population was cultured in a medium containing streptomycin

Table 3. Changes in rates of nitropropanol metabolism and most probable numbers of nitropropanol metabolizing and total bacteria in enrichment cultures^a.

Medium	Rate of nitropropanol metabolism ^c	MPN ^b	
		Nitropropanol metabolizing	Total
A ^d	0.52 ± 0.02	1.1 X 10 ⁸	2.4 X 10 ⁹
A', ^d	0.40 ± 0.01	1.1 X 10 ⁷	1.1 X 10 ⁸
B ^d	0.45 ± 0.03	1.1 X 10 ⁸	>1.1 X 10 ⁹
B ^e		2.4 X 10 ⁵	1.5 X 10 ⁸

^aSecondary enriched population, as described in text was cultured in medium A, which contained 40% clarified rumen fluid; medium A', which was the same as A but also contained 20 ug streptomycin/ml; or medium B, which was the same as A except it contained 8% instead of 40% clarified rumen fluid. Media was supplemented with 33.6 mM (medium A and A') or 67.2 mM (medium B) nitropropanol.

^bMPN; most probable number (organisms/ml) estimates were determined using three tube tests as described. MPN of nitropropanol metabolizing bacteria in ruminal fluid from cow not exposed to nitropropanol (inoculum before enrichment) was 3.0 X 10⁴ organisms/ml.

^cμmol/ml per h. Rates were determined after 48 h incubation. Values are the mean ± SD from two cultures.

^dMPN was determined on 48 h old cultures.

^eMPN was determined on 120 h old culture.

(Table 3). Thus, this strategy was not pursued further.

Rates of nitropropanol metabolism and the MPN differed little between 48 h old secondary enriched populations cultured in medium A or medium B (containing 8% instead of 40% rumen fluid) (Table 3). However, after incubation for 120 h in medium B, the MPN of both nitropropanol metabolizing

and total bacteria decreased, although the MPN of nitropropanol metabolizing bacteria decreased more than the MPN of the total population.

Preliminary studies indicated that H_2 was a possible substrate for nitropropanol metabolizing bacteria as rates of nitropropanol metabolism increased more rapidly when mixed populations were cultured consecutively under $H_2:CO_2$ (1:1) than under CO_2 (not shown). In support of this, the rate of nitropropanol metabolism was also higher when the secondary enriched population was grown in medium B plus 33.6 mM nitropropanol under $H_2:CO_2$ (0.40 ± 0.01 $\mu\text{mol/ml per h}$) than under CO_2 , $CO_2:CH_4$ (1:1), or H_2 (0.18 ± 0.01 , 0.14 ± 0.01 , and 0.05 ± 0.02 $\mu\text{mol/ml per h}$, respectively). Whether nitropropanol metabolizing bacteria were inhibited by an alkaline pH that would have resulted when grown under 100% H_2 in the CO_3 buffered medium was not discerned.

When the secondary enriched population was serially cultured in medium B supplemented with 8.4 to 25.2 mM nitropropanol and rumen fluid that had been depleted of endogenous energy supply, rates of nitropropanol metabolism were maintained, averaging 0.43 ± 0.08 $\mu\text{mol/ml per h}$ for at least 8 consecutive incubations. With phytone deleted from the medium; however, rates decreased markedly (Table 4), and the population failed to grow after the fifth successive incubation. Based on microscopic examination, the enriched

Table 4. Rates of nitropropanol metabolism and most probable numbers of bacteria during serial culture of the secondary enriched population in depleted rumen fluid medium lacking phytone.

Culture series ^b	Rate of nitropropanol metabolism ^c	MPN ^a		Age of culture
		Nitropropanol metabolizing	Total	
1st	.29	ND ^c	ND	-
2nd	.12	4.6 X 10 ⁷	2.4 X 10 ⁸	72 h
3rd	.21	7.3 X 10 ⁵	>1.1 X 10 ⁷	48 h
		1.5 X 10 ⁸	1.5 X 10 ⁸	96 h
4th	.03	ND	ND	-

^aMPN; most probable numbers (organisms/ml) were determined from the cultures at the ages indicated.

^bEach culture contained medium B modified by substituting clarified rumen fluid with energy depleted rumen fluid and omitting phytone. The secondary enriched population used to inoculate the first culture is described in the text. The first culture contained 8.4 mM nitropropanol and subsequent cultures contained 25.2 mM nitropropanol.

^cμmol/ml per h.

^dND; not done.

population grown in depleted rumen fluid medium lacking phytone was considerably less diverse than the population grown with phytone and MPN determinations revealed that in the third consecutive culture, nitropropanol metabolizing bacteria were among the most numerous (Table 4). The population contained in the MPN tube that had been inoculated with the highest dilution of the 96 h old culture (10⁻⁸ ml)

was maintained by serial transfer in medium B plus 25.2 mM nitropropanol. Rates of nitropropanol metabolism by this population (enriched population C) averaged 0.32 ± 0.08 $\mu\text{mol/ml}$ per h after 8 consecutive 48 h incubations.

Nitropropanol metabolizing bacteria in a 48 h culture of enriched population C were again among the most numerous with nitropropanol being metabolized in all tubes containing growth (MPN = $4.6 \times 10^8/\text{ml}$). Upon microscopic evaluation, we observed a population consisting of gram positive irregular rods (0.5 to 1.0 x 0.5 to 1.5 μm). Some of the rods were pear shaped while others were symmetrical. Chains were present. The larger gram positive rods (0.5 to 1 x 1 to 2.5 μm) that had been numerous in cultures prior to this step were not observed.

Isolation of a competent nitropropanol metabolizing bacterium. A nitropropanol metabolizing bacterium was subsequently isolated from enriched population C. After 120 h incubation, 27 colonies were picked from a roll tube inoculated with 10^{-4} ml of enriched population C (grown 48 h in medium B plus 25 mM nitropropanol). Only one of the 27 picks had grown after 96 h incubation and this isolate metabolized nitropropanol. This isolate (strain NPOH1) grew when incubated in medium B supplemented with nitropropanol or nitrate but growth was not observed, even after 5 d, in medium B supplemented with nitrite, fumarate or sulfate (each

at 4.2 mM). To insure purity, additional isolations of strain NPOH1 were carried out in succession. A single colony from each isolation was the source for the next; however, other colonies were picked and cultured to determine if they metabolized nitropropanol. The roll tube media used in the last isolation attempt was supplemented with 2.5 mM nitrate instead of nitropropanol, which allowed for the development of larger colonies. All five and all six colonies picked during the last two isolations metabolized nitropropanol and strain NPOH1 was thus deemed pure.

Discussion

This is the first report of isolation of a nitropropanol metabolizing bacterium from ruminal populations enriched for enhanced rates of nitropropanol metabolism. Enriched populations were obtained when microbes in bovine ruminal contents were cultured consecutively in a medium containing nitropropanol. That the enhanced rates obtained during consecutive batch culture reflected an enrichment of nitropropanol metabolizing bacteria was confirmed by most probable number estimates.

We suggest that enhanced rates of ruminal nitropropanol metabolism by cattle fed sublethal amounts of milkvetch or diets supplemented with soybean meal (7) reflect a selection of competent nitropropanol metabolizing bacteria. Results

from our consecutive batch studies are consistent with this hypothesis. For instance, rates of nitropropanol metabolism were enhanced when mixed populations were cultured consecutively in media containing milkvetch or alfalfa plus synthetic nitropropanol, but not in media containing alfalfa lacking nitropropanol. This suggests that selective pressure was exerted by nitropropanol. Our results also indicate that phytone is important for the growth of nitropropanol metabolizing bacteria since enhanced rates of nitropropanol metabolism were achieved more quickly and maintained at higher levels when populations were cultured in media containing phytone.

The nitropropanol metabolizing bacteria appeared to utilize H_2 as a reductant and this has now been confirmed in subsequent studies with strain NPOH1 (1). Results from studies with strain NPOH1 showed that nitrate can also be utilized as an electron acceptor. The ability of nitropropanol metabolizing bacteria to use nitrate may explain the enhanced rates of nitropropanol metabolism observed when cattle were fed diets containing nitrate (4).

More rapid rates of ruminal nitropropanol are also associated with cattle receiving orchardgrass rather than alfalfa (10). We found that ovine ruminal microbes metabolized nitropropanol and nitropropionic acid; however, we can not explain why rates were not increased when sheep

were fed nitrate, nitroethane, or orchardgrass.

Studies with various enriched populations provided evidence that nitropropanol metabolizing bacteria either compete poorly with other members of the mixed population for fermentable carbohydrates or do not use these substrates. Rates of nitropropanol metabolism by the enriched populations were not stimulated by Fe^{2+} and S^{2-} , as was the case with non-enriched populations (2). This suggests that nitropropanol is metabolized differently by the two populations.

Some characteristics of the nitropropanol metabolizing bacteria are reported in a companion paper (1). Our objective is that such work will enable us to develop better methods for isolation and quantitation of these bacteria and for evaluation of their role in the rumen. We hope that gaining an understanding of ruminal nitro-toxin metabolism will ultimately enable the development of strategies to prevent livestock poisonings by the toxins.

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**CHAPTER 3. CHARACTERISTICS OF A NITROPROPANOL METABOLIZING
BACTERIUM ISOLATED FROM THE RUMEN**

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Abstract

We report some characteristics of a ruminal bacterium (strain NPOH1) which metabolizes 3-nitropropanol, the toxic principle of various milkvetchs consumed by livestock. Strain NPOH1 is a gram positive, non-motile anaerobe. Spores were not detected. Growth of strain NPOH1, in a rumen fluid based medium was supported by the electron acceptors 3-nitropropanol, 3-nitropropionic acid, nitrate, 2-nitropropanol, nitroethane, nitroethanol, or 3-nitro-1-propyl- β -D-glucopyranoside (miserotoxin). Nitrate was the preferred acceptor when strain NPOH1 was grown in medium containing

both nitrate and 3-nitropropanol. Nitrate was reduced to nitrite by strain NPOH1 and when grown with excess reductant, the nitrite was further reduced to ammonia. Sulfate, sulfite, azide, chlorate, perchlorate, nitrite, fumarate, 2-nitrobutane, or nitrobenzene did not support growth. In the presence of appropriate nitrocompound, formate, lactate, and H_2 stimulated growth of strain NPOH1 whereas a variety of other potential H_2 donors did not. Our evaluations of many phenotypic properties including total cellular fatty acid profiles and of the 16S rRNA gene sequence of strain NPOH1 support the concept that this organism is a new species. The capacity of strain NPOH1 to metabolize 3-nitropropanol suggests that this organism may be an important 3-nitropropanol detoxifying bacterium.

Introduction

The toxins, 3-nitro-1-propanol and 3-nitro-1-propionic acid are constituents of many forages consumed by ruminants. *Astragalus* species containing 3-nitropropanol cause livestock poisonings; in North America, poisonings occur particularly in the Rocky Mountain regions (17, 18).

Acquisition of tolerance by animals consuming *Astragalus* occurs and is dependant on rates at which the nitro-toxins are metabolized by ruminal microbes as well as upon rates of toxin absorption across the rumen epithelium. Thus, the

nitro-acid, which is metabolized much more rapidly and absorbed more slowly than the nitro-alcohol, is less toxic than 3-nitropropanol (26). Since tolerance depends on rates of ruminal nitro-toxin metabolism, information about the organisms primarily responsible for this detoxification is critical for the design of practical strategies to prevent poisonings.

A screen of 63 strains of bacteria from culture collections of rumen organisms revealed that 19 strains metabolized 3-nitropropanol or 3-nitropropionic acid (23, 24). However, the rates at which these bacteria metabolized the nitrocompounds was considerably slower than rates by mixed ruminal populations (23), suggesting that organisms other than those tested are more competent and are primarily responsible for metabolizing the toxins. Rates of ruminal 3-nitropropanol metabolism are increased by feeding cattle a less toxic analog of 3-nitropropanol, nitroethane, or by feeding sublethal amounts of milkvetch (*Astragalus miser*) (22) suggesting that increased proportions of 3-nitropropanol metabolizing microbes are selected under these conditions.

In a companion paper (3), we reported the first successful isolation of a 3-nitropropanol metabolizing bacterium from populations enriched for enhanced rates of 3-nitropropanol metabolism. We report here some characteristics of this organism.

Materials and Methods

Organisms. Strain NPOH1 was isolated from a ruminal population enriched by consecutive culture in media containing 3-nitropropanol (3). Strain NPOH1 was designated strain NP1 in an abstract submitted to the 95th General Meeting of the American Society of Microbiology. Working cultures were maintained by serial transfer in medium B plus 3-nitropropanol, 3-nitropropionic acid, or nitrate. *Coriobacterium glomerans* (ATCC 49209 = DSM 20642) was obtained from the American Type Culture Collection. *Megasphaera elsdenii* B159, obtained from M. P. Bryant, and *Propionibacterium avidum* (ATCC 25577) were available in our culture collection.

Media and culture methods. For routine culture and for most tests, strain NPOH1 was grown in medium B (3) supplemented with 9 mM 3-nitropropanol, 9 mM 3-nitropropionic acid or 2.5 mM nitrate. When indicated, concentrations of these supplements were varied and/or other substrates were supplied. Medium B was prepared under an $H_2:CO_2$ (1:1) atmosphere unless indicated otherwise. When N_2 was used, Na_2CO_3 was deleted from medium B. General maintenance medium (GM) was the same as the basal medium used by Rasmussen (30) except 2-deoxyribose was omitted and glucose, cellobiose, soluble starch, xylose (each at 0.1% wt/vol) and trypticase (0.05% wt/vol) were added. Peptone-yeast extract medium (16)

was modified (MPYG) by omitting trypticase and adding 0.5% glucose, 0.2% phytone peptone, 0.05% $(\text{NH}_4)_2\text{SO}_4$, 0.17% acetic acid, 0.06% propionic acid, 0.04% n-butyric acid, 0.01% (each) n-valeric acid, isovaleric acid, isobutyric acid, and DL-methylbutyric acid. Complete defined medium was prepared by omitting phytone and rumen fluid from medium B and adding (mg/100 ml) L-alanine, 10; L-arginine, 24; L-asparagine, 40; L-aspartic acid, 10; L-glutamic acid, 30; glycine, 10; L-histidine, 6; L-isoleucine, 13; L-leucine, 13; L-lysine, 13; L-methionine, 5; L-phenylalanine, 5; L-proline, 10; L-serine, 3; L-threonine, 10; L-tryptophan, 2; L-tyrosine, 10; L-valine, 13; adenine, 1; guanine, 1; uracil, 1; xanthine, 1; hemin, 0.5; and volatile fatty acids as in MPYG. Brain heart infusion (BHI) medium (Difco) supplemented with 16 mM formate was prepared anaerobically under $\text{H}_2:\text{CO}_2$ (1:1) with the addition of 0.4% Na_2CO_3 , 0.025% cysteine, and 0.0001 resazurin. *M. elsdenii* was grown in yeast extract-lactate (YEL) medium which was prepared under 100% CO_2 and contained 12 g yeast extract, 2 g sodium lactate, 0.4 g Na_2CO_3 , 0.025 g cysteine-HCl, and 0.0001 g resazurin in 100 ml tap water. Trypticase-Phytone-yeast extract (TPY) medium (31) was used to test the ability of *C. glomerans* to grow in medium containing 3-nitropropanol, 3-nitropropionic acid, and nitrate. Media (4.5 or 9 ml) were dispensed under O_2 free gas into 18 X 150 mm tubes, sealed with rubber stoppers, and

autoclaved in a press. The final pH of medium B, MPYG, modified BHI, and YEL media was approximately 6.8; the final pH of TPY medium was approximately 6.5. For growing larger volumes of cells, medium was dispensed (400 ml) into side arm flasks and bubbled aseptically with gas after autoclaving or 12 liter volumes were prepared in a model SF-116 fermentor, (New Brunswick Scientific Company, Inc., Edison, NJ). Test compounds were added as small volumes (<3% vol/vol) from concentrated anaerobic stock solutions. Anaerobic techniques (7) were used except for the oxygen sensitivity test, when air or O₂ (100%) was injected through the rubber stoppers.

Strain NPOH1, *M. elsdenii*, and *P. avidum* were grown at 39°C; *C. glomerans* was grown at 30° C. Growth was measured as absorbance (600 nm) in 18 mm culture tubes (Spectronic 70 spectrophotometer, Bausch and Lomb Inc., Rochester, NY). Suspensions of cells of strain NPOH1, when corrected to A₆₀₀ = 1.0, had a dry weight of 179 µg/ml. Antibiotic resistance, determined by the broth dilution method, and the test for catalase were performed as described by Holdeman et al. (16) except that medium B, supplemented with 3-nitropropanol or nitrate, was used. Likewise, medium B containing 3-nitropropanol and 5 mM formate, supplemented with 0.1% L-tryptophan or with 0.015% ferrous sulfate and 0.015% sodium thiosulfate, was used to test for the production of indole (method 3) and H₂S (method 1) (34); medium B plus 3-

nitropropanol, 5 mM formate and 4% gelatin was used to test for gelatin hydrolysis (34). All experiments were performed with duplicate cultures. API-ZYM, AN-IDENT, and API-20A test kits were obtained from Analytab Products (Plainview, NY).

Microscopy. Microscopy was conducted as described by Cornick et al. (10). Cells stained for the presence of spores (Schaeffer-Fulton method; 12) were obtained from cultures that had been incubated one week in the following media supplemented with 8 to 9 mM 3-nitropropanol: egg yolk agar and cooked meat medium (Difco, each prepared under 100% H_2); the sporulation medium of Skarma and Hobson (33); and in medium B. These cells were also tested for heat tolerance by heating at 80°C for ten min (16). Cells grown as above in medium B for 2 and 7 wk were also stained for spores. Stains for poly- β -hydroxybutyrate and polyphosphate as well as both the Periodate-Schiff and Alcian blue stains for polysaccharides were performed as described by Doetsch (12).

Analytical methods. Concentrations of ammonia (9), nitrite (32), 3-nitropropanol and 3-nitropropionic acid (4) were determined colorimetrically. Selected culture fluids were analyzed for aminopropanol and β -alanine by high performance liquid chromatography and thin layer chromatography as previously described (4). Protein concentrations were determined using the modified Lowry procedure (28) with bovine serum albumin used as standard.

Concentrations of volatile fatty acids and alcohols were determined by gas liquid chromatography (GLC; 1). Formate, nitrate, and nitrite were also measured by anion exchange chromatography (DX-100 Ion Chromatograph, Dionex Corp., Sunnyvale, CA). Sample fluids (25 μ l) were eluted at a flow rate of 2 ml/min through an Ionpac AS4A column (4 mm X 250 mm). The mobile phase was 1 mM Na_2CO_3 in 0.85 mM NaHCO_3 and ions were measured by an ion conductivity detector. Gas production was measured by gas chromatography (series 580 Thermal Conductivity gas chromatograph; Gow-Mac Instrument Co., Bound Brook, NJ) with nitrogen or helium as the carrier gas when analyzing for hydrogen or carbon dioxide, respectively. A gas sampling valve was used to inject 0.5 ml of headspace gas. Total cellular fatty acids were determined by GLC of methyl esters using methods described by Miller (27), except cells were grown in medium B supplemented with 3-nitropropanol, 3-nitropropionic acid, nitrate, or in BHI supplemented with nitrate. Identities of acids given are those designated by the Microbial Identification System software package (Microbial ID, Inc., Newark, DE). These identifications are based solely on retention times relative to standards during temperature programmed GLC and further study would be required for positive designations.

DNA extraction and G + C determentation. Base guanine + cytosine (G + C) composition was determined by the

thermal melt method (19) with reference T_m obtained using DNA from *E. coli* and *P. avidum*. DNA from strain NPOH1 was extracted from cells grown in medium B supplemented with 16 mM formate and nitrate, DNA from *P. avidum* was extracted from cells grown in MPYG. *E. coli* DNA was provided Dr. T. B. Stanton and its preparation has been described previously (35). Cells of strain NPOH1 and *P. avidum* were incubated with 1 mg Penicillin G/ml for 4 h prior to harvest. Cells were washed 1X in 10 mM Tris plus 0.85% NaCl (pH 8.0), resuspended in 1.6 ml TE buffer (10 mM Tris plus 1 mM EDTA, pH 8.0) and then incubated 1 h at 39°C with 2 mg lysozyme/ml. Proteinase K (0.2 mg), 0.9% N-lauroylsarcosine, and 0.06 M EDTA were added to the cell suspensions and these were stored at 4°C overnight. The solutions were deproteinized by extracting 2X with Tris buffered phenol/chloroform (1:1) pH 8.5. Sodium acetate (0.3 M) and ethanol (0.6% vol/vol) were added to each solution and the precipitated DNA was spooled on to a pipet and redissolved into buffer (0.06% NaCl plus 0.04% trisodium citrate; pH 7.2) and incubated at 42°C overnight with 0.1 mg RNAase. After a final chloroform extraction, the DNA was again spooled and dissolved as above.

Ribosomal RNA. Copies of the 16S rRNA gene of strain NPOH1 were obtained by polymerase chain reaction (PCR) amplification. The forward primer 5-GAGTTTGATC(C/A)TGGCTCAG-3 and reverse primer 5-GGTTACCTTGTTACGACTT-3, corresponding

to positions 9-27 and 1510-1492, respectively, in the *E. coli* 16S rRNA gene (6), were used to amplify the gene. The reaction mixture contained 20 ng of DNA from strain NPOH1, 2.5 mM MgCl₂, 0.4 mM dNTP mix, 0.25 μM of each primer, and 2.5 U Taq polymerase (Amplitaq, Perkin Elmer Cetus, Norwalk, CT) in a total volume of 80 μl PCR buffer (Perkin Elmer Cetus). A wax barrier (Ampliwax PCR Gem 100, Perkin Elmer Cetus) separated the Taq polymerase and the primers from the other reaction components until the first (hot start) cycle. Amplification proceeded with an initial hot start (98°C, 2 min) followed by 30 cycles of denaturation (95°C, 1 min), annealing (48°C, 1 min) and synthesis (72°C, 2 min) and one last cycle with an extended synthesis step (8 min). The reaction mixture was then cooled (10°C) for 60 min. The PCR product was harvested and washed twice (with distilled H₂O) by ultra-filtration using a Micron 100 (Amicon, Inc., Beverly, MA) which was centrifuged 3000 x g for 10 min. DNA concentrations and size of the DNA products were estimated by staining intensity of bands after electrophoresis (1% agarose) of a portion of the clarified product. Sequencing was performed as described by Frothingham et al. (13). A partial sequence of the 16S rRNA gene product was obtained using in addition to the primers used for amplification, the forward primer 5'-ATTAGATACCCTGGTAG-3' and the reverse primer 5'-CCGTCAATTCATTTGAGTTT-3' corresponding to positions to 786-

803 and 926-907, respectively, on the 16S rRNA gene of *E. coli* (6). Two additional forward primers, 5'-CACATTGGGACTGAGATAC-3' and 5'-ACCCGGTGGCCGAGAG-3', and a reverse primer, 5'-GCTCCCCCACACCTAGT-3', were synthesized and used to determine remaining sequences of the 16S rRNA gene of strain NPOH1. These sequences correspond to positions 311-329, 1015-1030, and 840-824 on the 16S rRNA gene of *E. coli* (6). The 16S rRNA gene sequence of strain NPOH1 was compared to sequences in GenBank (5) using the Basic Local Alignment Search Tool (2).

Preparation of membrane fractions. Cells grown in medium B plus 9 mM 3-nitropropionic acid and 16 mM formate under CO₂ were harvested using a Sharples continuous flow centrifuge (Philadelphia, PA). Four g (wet cell weight) of cells were washed and resuspended using 100 mM potassium phosphate buffer (pH 6.8). The cell suspension was passed (4X) through a French pressure cell (20,000 lb/in²) and after removal of cell debris by centrifugation (10,000 x g, 10 min), the cell-free extract was retained. A portion of the cell-free extract was centrifuged at 170,000 x g for 1 h. The resultant supernatant (soluble fraction) was removed and the pellet (membrane fraction) was resuspended in 1 ml 100 mM potassium phosphate buffer (pH 6.8). Centrifugation steps were at 0 to 4°C and fractions were stored at -80°C until they were examined by absorption spectroscopy with an SLM

Aminco DW-2000 spectrophotometer in the split beam mode (36). A portion of the membrane fraction was combined (1:1) with 1000 mM KCl and centrifuged at 170,000 x g for 1 h. Absorption spectroscopy was again performed on both the supernate and pellet (resuspended in 100 mM potassium phosphate buffer, pH 6.8) to determine whether the cytochromes were strongly bound to the membranes.

Measurements with washed cells. Strain NPOH1 was grown to mid-late log phase in 400 ml of medium B supplemented with 16 mM formate and with 3-nitropropanol (9 mM), 3-nitropropionic acid (9 mM), or nitrate (2.5 mM). *M. elsdenii* was grown to mid-log phase in 12 liters of YEL medium. Cells were harvested by centrifugation (18,000 x g, 10 min), washed once and resuspended using anaerobic dilution solution (7). Activity was determined by incubating the washed cells in 13 x 100 mm tubes in 1.5 ml anaerobic dilution solution plus 3-nitropropanol, 3-nitropropionic acid, or nitrate (each at 20 mM). The reductant was provided as $H_2:CO_2$ (1:1).

Chemicals. Miserotoxin was provided by Dr. Walter Majak (Kamloops, B.C., Canada); 3-nitropropanol was purchased from Chem-Biochem Research Inc. (Salt Lake City, UT). Other nitrocompounds were purchased Aldrich (Milwaukee, WI.). Antibiotic discs were purchased from BBL (Becton Dickinson Microbiology Systems, Cockeysville, MD). All other chemicals

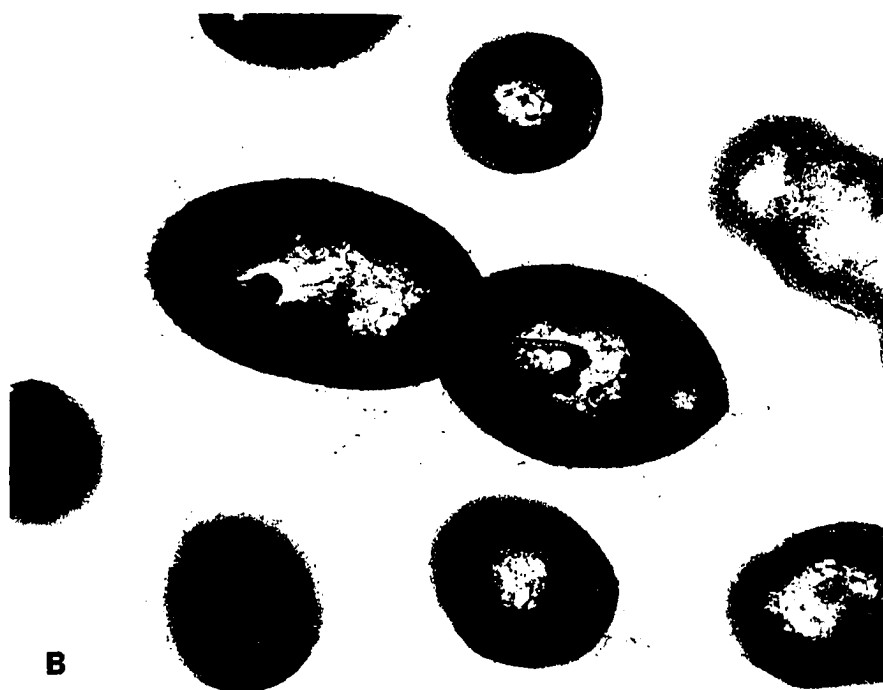
used were of analytical grade.

Results

Morphology. Cells of strain NPOH1 were rod shaped (0.5-1.0 X 1.0-2.0 μm) and some cells had irregular ends thus appearing bulbous. Cells stained gram-positive, did not react with 3% KOH to produce a slimy interface, as typically observed with gram-negative cell types (14), and examination of thin sections under an electron microscope revealed gram-positive cell wall structures (Figure 1). Flagella were not observed. Examination of thin sections also revealed the presence of inclusion bodies which were more frequent in 3-nitropropanol grown cells than in 3-nitropropionic acid or nitrate grown cells (Figure 1). The inclusion bodies could be seen in negatively stained cell preparations as well, suggesting that the bodies were electron dense. Stains for known types of inclusion bodies such as poly- β -hydroxy butyrate, glycogen, starch or polyphosphate granules were all negative. Likewise, no evidence for the existence of spores was found when cells stained for spores were examined by light microscopy or when they were tested for heat tolerance.

DNA characteristics. Mole percent of G + C of DNA from isolate NPOH1 was 60.6 ± 0.6 mole percent (mean \pm SD from determinations made on DNA from two separate extractions). The closest match (86% homology) found between

Figure 1. Electron micrographs of cells of strain NPOH grown in medium B containing nitropropanol. A, cells stained with 0.5% phosphotungstic acid (bar = 0.34 μm). B, thin section showing typical gram-positive cell wall (bar = 0.25 μm).

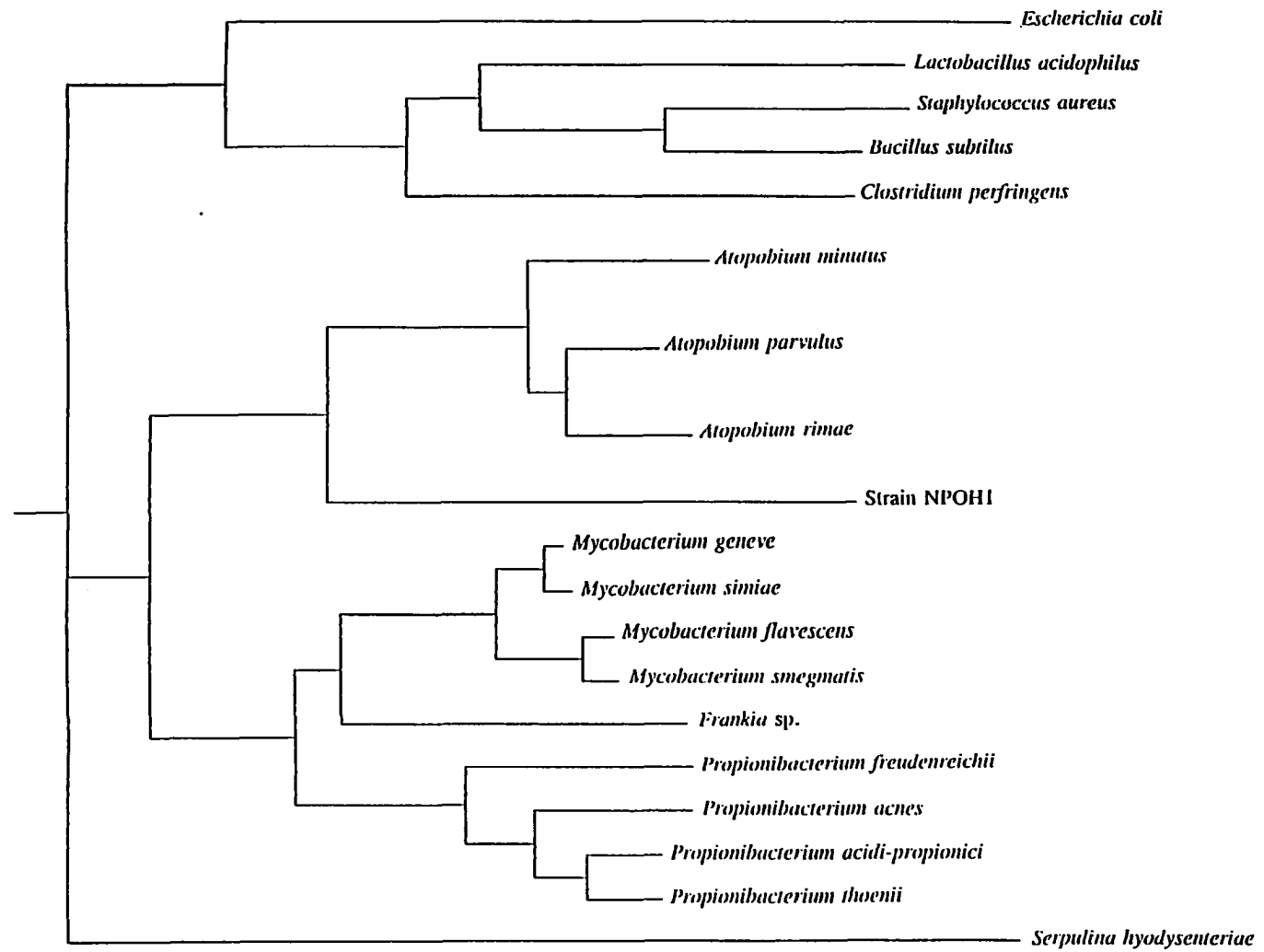


the 16S rRNA gene sequence of strain NPOH1 (Table 1) to sequences available in GenBank (5) was that of *C. glomerans* (accession number X79048). The phylogenic relationship of strain NPOH1 to some other microorganisms is depicted in Figure 2. Tests for 3-nitropropanol and 3-nitropropionic acid metabolism in medium that supported dense growth revealed that *C. glomerans* did not grow when these

Table 1. Nucleotide sequence of the 16S rRNA gene of strain NPOH1.

1	CGCTGGCGGC	GTGCCTAACA	CATGCAAGTC	GAACGAATAA	CCCGCCTTCG
51	GGCGGTCATA	GAGTGGCGAA	CGGGTGAGTA	ACACGTGACC	AACCTTCCCC
101	CCGCATGGGG	ATAACCGGGC	GAAAGCCCGG	CTAATACCCA	ATACTCCGGG
151	CCCTCCGCAT	GGAGGGGCCG	GGAAAGCCCA	GGCGGCGGGG	GATGGGGTCG
201	CGGCCCATTA	GGTAGACGGC	GGGGTAACGG	CCCACCGTGC	CCGCGATGGG
251	TAGCCGGACT	GAGAGGTCGA	TCGGNCACAT	TGGGACTGAG	ATACGGCCAG
301	ACTCCTACGG	GAGGCAGCAG	TGGGNAATTT	TNCGCNATGG	GNNAAACCTT
351	GAGCNGCNA	CGCCGCGTGC	GGGATGAAGG	CCCTAGGGTT	GTAANCCNCT
401	TTCNGCNGGG	AAGAAACNTG	ACCGNACCTG	CAGAAGAAGC	TCCGNCCTAAC
451	TACGTGCCAG	CAGCCGCGGT	AATACGTAGG	GAGCGAGCGT	TATCCGGATT
501	CATTGGGCGT	AAAGCGCGCG	TAGGCGGCTG	NCCAANCGGG	ATCTCANATC
551	CGGGGGGCTCA	ACCTCCGGCC	GGATCCCGAA	CTGNCCGGCT	CGAGTTCGGT
601	AGAGGAAGAC	GGAATTCCCA	GTGTAGCGGT	GNAATNCGCA	GATATTGGGA
651	AGAACACCGA	TGNCGAAGGC	AGTCTTCTGG	GCCNCGACTG	ACNCTGAGGT
701	GCGAAAGCCG	GGGGAGCGAA	CAGGATTAGA	TACCCTGGTA	GTCCCGCCCCG
751	TAAACGATGG	GCACTAGGTG	TGGGGGAGCC	TTTCCTCCGC	GCCGCAGCTA
801	ACGCATTAAG	TGCCCCGCCT	GGGGAGTACG	GCCGCAAGGC	TAAAACTCAA
851	AGGAATTGAC	GGGGGCCCGC	ACAAGCAGCG	GAGCATGTGG	NTTAATTCTGA
901	AGCAACGCGA	AGAACCTTAC	CAGGGCTTGA	CATGCGGGTG	AAGCCGGGGA
951	AACCCGGTGG	CCGAGAGGAG	CCCGCGCAGG	TGGTGNATGG	CTGTCGTCAG
1001	CTCGTGTCGT	GAGATGTTGG	GTTAAGTCCC	GCAACGAGCG	CAACCCCTGC
1051	CCCATGTTGC	CAGCATTAGG	TTGGGGACTC	ATGGGGGACT	GCCGGCGTCA
1101	AGCCGGAGGA	AGGTGGGGAC	GACGTCAAGT	CATCATGCCC	CTTATGCCCT
1151	GGCCCGCACA	CGTGCTACAA	TGGCCGGTAC	AGAGGGCTGC	CAGACCGCGA
1201	GGTCGAGCGA	ATCCCCCAA	GCCGGCCCCA	GTTCCGACAG	GAGGCTGCAA
1251	CCCGCCTCCT	CGAAGCCGGA	GTTGCTAGTA	ATCGCGGATC	AGCACGCCGC
1301	GGTGAATACG	TTCCCGGGCC	TTGTACACAC	CGCCCGTCAC	ACCACCCGAG
1351	TCGTCTGCAC	CCGAAGTCCC	CGGCCCAACC	TCGTGAGGGA	GGGGCCGAAG
1401	GTGTGGAGGG	TGA			

Figure 2. Phylogenic relationship of strain NPOH1 to some other bacteria based on analysis of the 16S rRNA gene sequence.



nitrocompounds were added (5 mM). *C. glomerans* grew with 5mM nitrate but the amount of growth was equal to that when grown without nitrate.

Cellular Fatty Acids. Profiles of total cellular fatty acids of strain NPOH1 differed somewhat depending upon growth conditions (Table 2). Fatty acid methyl esters containing 14 or 15 carbons were predominant in cells grown in medium B supplemented with nitrate, 3-nitropropionic acid or 3-nitropropanol; however, esters of other lengths, including hydroxy acids, were present. The methyl ester of palmitic acid (16:0) was predominant in cells grown in BHI medium supplemented with nitrate (not shown) but esters of other acids and hydroxy-acids were again present. When a computerized search was conducted to compare profiles of methyl esters from strain NPOH1 with those of organisms in the MIDI VPI Anaerobe Library and Aerobe Library data bases, no matches were found.

Growth. Strain NPOH1 grew in medium B when electron acceptors such as 3-nitropropanol, 3-nitropropionic acid, nitrate, nitroethane (supplied as the sodium salt; 25), nitroethanol, 2-nitropropanol, or miserotoxin (3-nitro-1-propyl- β -D-glucopyranoside), each at 5 mM, were supplied. Growth was not supported by the acceptors azide, sulfate, sulfite, fumarate, nitrite, 2-nitrobutane, nitrobenzene, chlorate, or perchlorate (each at 5 mM), even after 120 h

Table 2. Total cellular fatty acid profiles of strain NPOH1.

Fatty acid	NPOH ^a	NPA	NO ₃
C10:0			
iso	3 (.4) ^b	2 (.3)	3 (.3)
C12:0			
iso	3 (.4)	3 (.4)	ND ^c
C13:0			
iso	13 (1.5)	19 (2.7)	ND
anteiso	ND	3 (.5)	ND
normal	7 (.9)	6 (.9)	ND
iso 3-hydroxy	7 (.8)	7 (1.0)	8 (.9)
C13:1			
at 12-13	17 (2.0)	15 (2.1)	11 (1.1)
C14:0			
iso	51 (6.0)	41 (5.7)	86 (8.7)
normal	100 (11.9)	74 (10.3)	62 (6.3)
iso 2-hydroxy	14 (1.6)	16 (2.2)	13 (1.3)
iso 3-hydroxy	48 (5.7)	63 (8.8)	6 (.6)
C15:0			
iso	96 (11.5)	100 (13.9)	15 (1.5)
anteiso	73 (8.7)	66 (9.1)	100 (10.2)
normal	31 (3.7)	21 (2.9)	8 (.8)
C16:0			
iso	19 (2.2)	10 (1.4)	49 (4.9)
normal	41 (4.9)	27 (3.7)	50 (5.1)
C18:0			
normal	14 (1.7)	9 (1.3)	12 (1.2)
C19:1			
iso	16 (1.9)	16 (2.2)	21 (2.1)

^aGrown in medium B with 9 mM nitropropanol (NPOH), 9 mM nitropropionic acid (NPA), or 2.5 mM NaNO₃ (NO₃).

^bvalue outside parenthesis is relative molar percentage with most abundant at 100%, value within parenthesis is percentage of total fatty acids.

^cND, not detected.

incubation. When grown in medium containing both nitrate and 3-nitropropanol, each at 5 mM, nitrate was metabolized to nitrite before 3-nitropropanol was metabolized. Strain NPOH1 grew poorly if at all in anaerobic media such as PYG or GM, whether supplemented with 3-nitropropanol or nitrate.

Strain NPOH1 required a complex medium component such as phytone peptone for continued growth during consecutive serial transfer. For instance, strain NPOH1 grew moderately upon initial culture in BHI or in complete defined medium, each supplemented with 5 mM nitrate and 16 mM formate (A_{600} 0.37 and 0.29, respectively), but could not be maintained upon successive transfers into either of these media.

Substitution of phytone in medium B containing 5 mM nitrate and 16 mM formate with equal amounts (0.8% wt/vol) of yeast extract or proteose peptone resulted in equivalent growth but substitution with trypticase, casamino acids, or acid or enzymatic hydrolysates of casein resulted in 50 to 75% less growth. Growth of NPOH1 in medium containing increasing amounts of phytone increased proportionally ($R^2=0.93$) up to 0.4%, but did not increase further with levels of up to 2%.

While strain NPOH1 grew in medium B under a 100% CO₂ gas phase, H₂ was stimulatory and growth was better with 50% H₂ in CO₂ than with 20% H₂. Growth of strain NPOH1 in medium B containing 3-nitropropanol (5 mM) or nitrate (2.5 mM) was also stimulated by formate or lactate but not by adonitol,

arabinose, cellobiose, dulcitol, erythritol, ethanol, fructose, galactose, galacturonic acid, gluconic acid, glucose, glycerol, inositol, lactose, malic acid, mannitol, mannose, maltose, melezitose, melibiose, methanol, methylamine, propanol, pyruvate, raffinose, rhamnose, ribose, salicin, sorbitol, succinate, sucrose, trehalose, or xylose (each at 20 mM).

The amount of growth by strain NPOH1 increased proportionally in medium containing increasing concentrations of electron acceptor (Figure 3) but at higher concentrations cell yields began to level off suggesting that other factors had become limiting. Cell yields increased when strain NPOH1 was grown in medium B containing a constant amount of nitrate provided that increasing amounts of reductant were supplied (Table 3) and similar results were observed with cells grown likewise with 3-nitropropanol or 3-nitropropionic acid (not shown). However, if calculated on the basis of yield of cells per mole of acceptor metabolized, the efficiency of growth decreased as the concentration of acceptor was increased. The maximum specific growth rates obtained during growth in medium B containing both $\text{H}_2:\text{CO}_2$ (1:1) and 16 mM formate were 0.11 h^{-1} , 0.12 h^{-1} , and 0.22 h^{-1} when grown with 9 mM 3-nitropropanol, 9 mM 3-nitropropionic acid or 2.5 mM nitrate, respectively.

Strain NPOH1 grew with up to 4% O_2 in the headspace gas

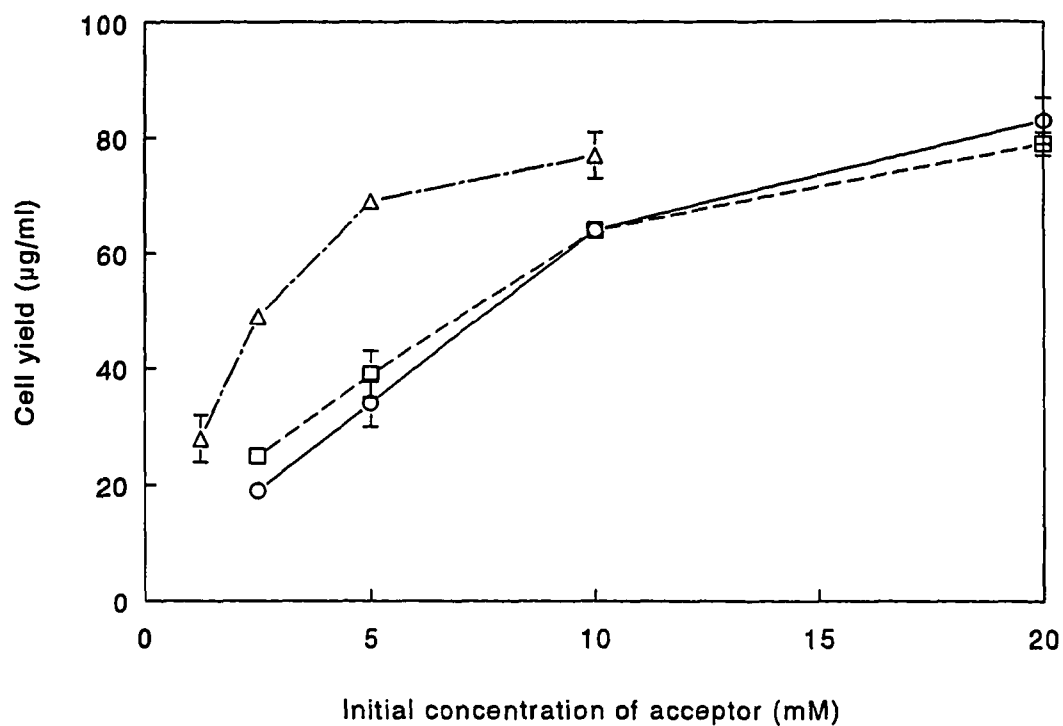


Figure 3. Cell yields of strain NPOH1 when grown in medium B plus increasing amounts of nitropropanol (circles), nitropropionic acid (squares), or nitrate (triangles). Values are the mean from duplicate cultures. SD were less than 4 µg/ml unless indicated otherwise.

Table 3. Disappearance of nitrate and accumulation of nitrite and ammonia during culture of strain NPOH1 in media containing varying amounts of formate and 10 mM nitrate.

Concn of formate in media ^c	<u>Lost^a</u>	<u>Recovered</u>		Cell yield ^b (μg dry wt/ml)
	NO ₃ (mM)	NO ₂ (mM)	NH ₄ (mM)	
With H ₂ :CO ₂				
0	6.2 ± 0.4	2.9 ± 0.4	3.4 ± 0.4	48.4 ± 5.4
16	10.3 ± 0.1	2.8 ± 0.2	8.2 ± 0.2	129.9 ± 2.7
With CO ₂				
16	6.6 ± 0.3	7.0 ± 0.3	4.1 ± 0.2	114.6 ± 1.8
68	8.5 ± 2.4	ND ^d	12.3 ± 0.4	197.9 ± 8.1

^aDifference between concentrations measured at 0 and 48 h. Values are the mean \pm SD from duplicates.

^bDry weight estimates were determined by correcting A₆₀₀ to dry weights. Suspensions at A₆₀₀ 1.0 had 179 μ g dry weight/ml.

^cCultures were grown in medium B under 100% CO₂ or H₂:CO₂ (1:1).

^dND; not detected.

if cysteine was present in the medium but did not grow with as little as 1% O₂ in the headspace gas when cysteine was deleted.

Antibiotic sensitivity. Strain NPOH1 was sensitive to cephalothin (6 μ g/ml), clindamycin (1.6 μ g/ml), chloramphenicol (12 μ g/ml), and erythromycin (3 μ g/ml) but was resistant to penicillin (2 U/ml), ampicillin (4 μ g/ml), carbenicillin (100 μ g/ml), and tetracycline (6 μ g/ml).

Metabolism. No evidence was found for the production of indole or H_2S by strain NPOH1. Catalase activity also was not detected and gelatin was not hydrolyzed. Strain NPOH1 produced a small amount of acetic acid ($< 1 \mu\text{mol/ml}$), as determined by the difference in acid concentrations at 0 and 28 h, when grown in medium B supplemented with 3-nitropropanol or nitrate but not with 3-nitropropionic acid. No acids were detected when strain NPOH1 was grown 96 h as above in medium which lacked rumen fluid. Measurements of pH in medium B supplemented with 3-nitropropanol showed that little, if any, acids were produced during growth of strain NPOH1 as pH was 6.79 ± 0.02 before inoculation and the pH was 6.92 ± 0.06 after 120 h incubation. When strain NPOH1 was grown 18 h under an N_2 atmosphere in medium B plus 16 mM formate and either 3-nitropropanol or 3-nitropropionic acid, equal quantities of H_2 and CO_2 were detected in the headspace (1% and 3% for cells grown with 3-nitropropanol or 3-nitropropionic acid, respectively), but these gases were not detected after growth with nitrate. Alcohols were not detected. When strain NPOH1 was grown with nitrate and limiting amounts of formate as reductant, nitrite accumulated in cultures. If grown with excess formate, the nitrite was further metabolized to ammonia (Table 3). Appreciable quantities of nitrite or ammonia did not accumulate in cultures grown with 3-nitropropanol or 3-nitropropionic acid

and aminopropanol and β -alanine were not detected.

Utilizing ANALYTAB methods, cells of strain NPOH1, grown in medium B plus 16 mM formate and 3-nitropropanol, exhibited positive reactions for arginine aminopeptidase (An-indent), phosphatase acid and Naphthol-AS-BI-phosphorohydrolase (API-ZYM) and weak positives for phosphatase (An-indent) and lipase-C14 (API-ZYM). All reactions were negative when cells were tested using the API-20A system, however, this test is based on growth in Lombard-Dowell medium during the 24 h incubation period and it is probable that strain NPOH1 failed to grow.

Measurements with washed cells. Cells of strain NPOH1 grown in medium B containing 16 mM formate and 3-nitropropanol actively metabolized 3-nitropropanol but not 3-nitropropionic acid (Table 4). Cells grown with nitrate metabolized nitrate stoichiometrically to ammonia; however, 3-nitropropanol and 3-nitropropionic acid were metabolized slowly. Conversely, cells grown with 3-nitropropionic acid metabolized both 3-nitropropanol and 3-nitropropionic acid. *Megasphaera elsdenii*, grown in YEL medium, had a specific 3-nitropropanol activity one-eighth ($0.3 \pm 0.1 \mu\text{mol/mg protein per hour}$) that of strain NPOH1.

Cytochrome C. Cell-free extracts of strain NPOH1 contained a c-type cytochrome with a α absorption maximum at 554 nm (Figure 4). The reduced absorption maxima was not

Table 4. Activities of washed cell suspensions of strain NPOH1 grown with nitropropanol, nitropropionic acid, or nitrate.

Acceptor during growth ^a	Specific activity ($\mu\text{mol}/\text{mg}$ protein/h)		
	Nitropropanol disappearance	Nitropropionic acid disappearance	Ammonia production
Nitropropanol	2.6 ± 0.1	NA ^b	NT ^c
Nitropropionic acid	$2.8 \pm <.1$	5.2 ± 0.9	NT
Nitrate	$0.1 \pm <.1$	$0.1 \pm <.1$	2.6 ± 0.1

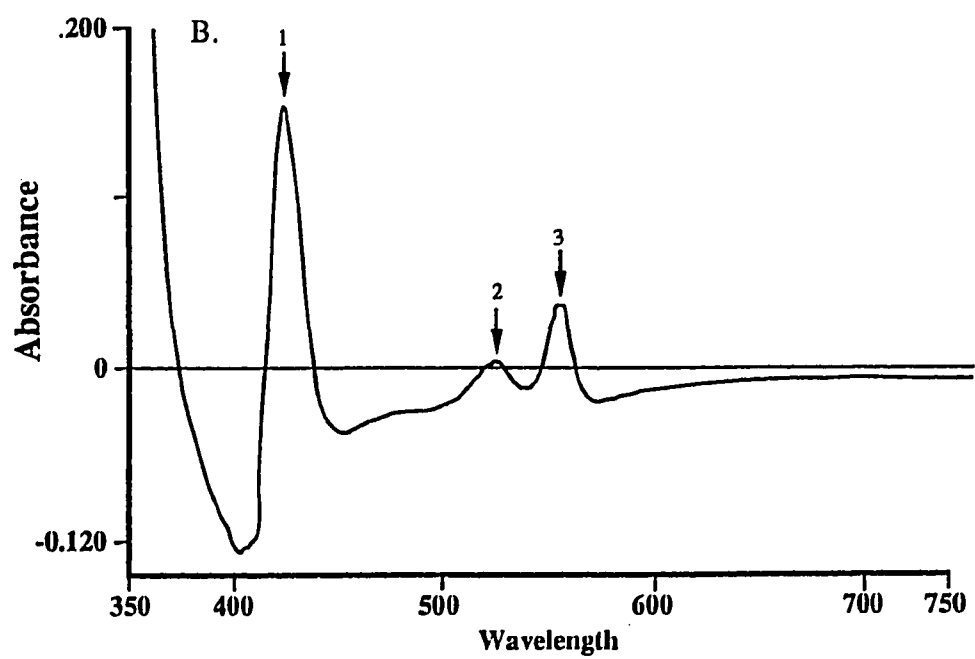
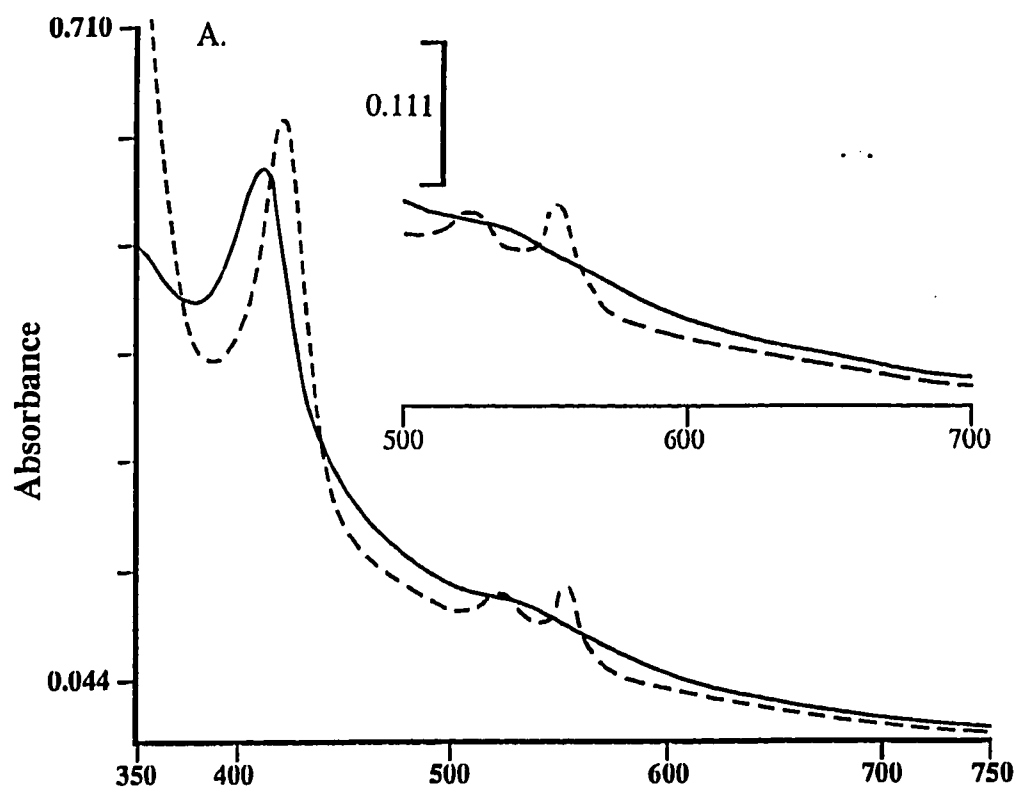
^a Activity was determined during incubation (8 h) of 0.3 mg cell protein of nitropropanol and nitropropionic acid grown cells or 2.1 mg cell protein of nitrate grown cells as described in materials and methods.

^bNA; no activity.

^cNT; not tested.

shifted by CO. Using the pyridine ferrohemochrome method (11), the heme content in the cell-free extract was estimated to be $1.3 \mu\text{mol}/\text{mg}$ protein. The pyridine ferrohemochrome absorption spectrum showed peaks at 414, 520, and 550 ± 1 nm indicative of c-type cytochromes. The cytochrome was present in both the soluble and membrane fractions; the cytochrome associated with the membrane fraction remained bound after treatment with high salt concentration (500 mM KCl).

Figure 4. Absorption spectra of membrane fraction containing *c*-type cytochrome. Cuvettes contained 1 ml 100 mM phosphate buffer (pH 6.8) and membrane fraction (0.5 mg protein) from cells grown with nitropropionic acid and 16 mM formate. A, resting (—) and dithionite reduced (- - -) spectra; B, reduced minus resting spectra.



Discussion

Our evidence suggests that strain NPOH1 obtains energy for growth by anaerobic respiration. For instance, growth did not occur unless a suitable electron acceptor was supplied and the amount of growth was directly related to amounts of 3-nitropropanol, 3-nitropropionic acid, or nitrate supplied. The presence of a c-type cytochrome is also consistent with the anaerobic respiration proposal. Presumably, strain NPOH1 does not ferment sugars since growth was not stimulated when these were supplemented to medium B. The strict requirement for a complex medium component such as phytone, which supported low levels of growth in the absence of added reductant, limited our ability to conclusively test for growth on sugars. Growth occurred in medium lacking the phytone, but consecutive serial transfers could not be maintained.

Our evidence obtained with growing cultures (Table 3) and with washed cells (Table 4) demonstrating that strain NPOH1 reduces nitrate to ammonia is not surprising since ammonia is the principle product of nitrate reduction within the rumen (20). The capacity of strain NPOH1 to reduce nitrate and nitrite was dependent on the availability of reducing equivalents as has been observed with other populations of ruminal microbes (21).

We previously found that mixed ruminal populations

reduced 3-nitropropanol to aminopropanol and 3-nitropropionic acid to β -alanine (4). These products were; however, not detected in supernatants of cultures or washed cell mixtures of strain NPOH1. Further work is needed to determine if the nitro-toxins are reduced incompletely to intermediate products or are metabolized to produce other, as yet unknown, products.

Strain NPOH1 differs from any other known H_2 , formate, or lactate utilizers from the rumen or other anaerobic habitats. Our evidence for uniqueness of strain NPOH1 is based on results of measurements and observations of many phenotypic properties as well as the comparison of the 16S rRNA gene sequence with sequences deposited in a large data bank (5). This evidence suggests that strain NPOH1 has not been described previously. We have, however, decided to postpone taxonomic placement of this organism until other isolates have been obtained and characterized.

The amounts of 3-nitropropanol metabolized (>20 mM) by strain NPOH1 during growth far exceed the amounts metabolized by other known 3-nitropropanol metabolizing organisms (23, 24). With H_2 as reductant, the specific activity of 3-nitropropanol metabolism by strain NPOH1 was also greater than that of *M. elsdenii* B159 (Table 3) or of mixed populations (not shown). Thus, bacteria represented by strain NPOH1 could be of particular importance in the

acquisition of tolerance of animals eating poisonous forage such as milkvetch. Whether or not organisms such as strain NPOH1 are responsible for the enhanced rates of 3-nitropropanol metabolism observed when cattle are fed supplements of nitroethane, nitrate, or sublethal quantities of *Astragalus miser* remains to be determined. Evidence (3) suggests that numbers of nitropropanol metabolizing microbes, present at approximately 10^3 cells/ml in rumen fluid collected from naive cows, could be increased to approximately 10^8 cells/ml during enrichment. Thus, it is reasonable to expect that organisms like strain NPOH1 could be maintained at increased numbers in the rumen as the result of dietary manipulation, and this may provide the host with a detoxification potential that will reduce the risk of poisoning by these nitro-toxins.

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CHAPTER 4. SUMMARY AND DISCUSSION

Ruminal microbes are known to metabolize nitropropanol and nitropropionic acid, the toxins present in many milkvetchs consumed by ruminants, and when rates of nitro-toxin metabolism are sufficiently rapid, animals can acquire tolerance to the poisons. This study was undertaken to define conditions which promote ruminal detoxification of the nitro-toxins and to isolate microbes that play a major role in this detoxification. Information gained from these investigations may be useful in developing methods of preventing nitro-toxin poisoning.

We report observations on the enrichment and isolation of a nitropropanol metabolizing bacterium from the rumen. Rates of nitropropanol metabolism increased when mixed populations of ruminal microbes were cultured consecutively in a rumen fluid based medium containing milkvetch forage or alfalfa forage supplemented with nitropropanol. When phytone was also included in the medium, rates increased even more rapidly and to a higher extent. Most probable number estimates confirmed that concentrations of competent nitropropanol metabolizing bacteria were increased during consecutive batch culture.

An anaerobic, gram-positive, non-motile nitropropanol metabolizing bacterium (strain NPOH1) was isolated from an enriched population. Growth of strain NPOH1 was dependent on

the presence of a protein component such as phytone and a suitable electron acceptor. Acceptors that supported growth were nitropropanol, nitropropionic acid, nitrate, nitroethane, 2-nitropropanol, nitroethanol, and miserotoxin. Azide, chlorate, perchlorate, fumarate, sulfate, sulfite, or nitrite did not support growth. Formate, H₂, or lactate stimulated growth of strain NPOH1 but any of a number of other potential energy substrates did not. When grown with excess reductant, the cell yields of strain NPOH1 increased proportionally to the supply of acceptor thus suggesting that energy for growth is obtained by respiratory processes. Membrane preparations of strain NPOH1 were found to contain a c-type cytochrome thus supporting the proposed respiratory energy metabolism of strain NPOH1.

Strain NPOH1 reduced nitrate to nitrite, and when grown with excess reductant, the nitrite was reduced to ammonia. The possible products of nitropropanol and nitropropionic acid reduction, aminopropanol and β -alanine were not detected.

The physiological evidence discussed above as well as evidence obtained during analysis of total cellular fatty acids suggests that strain NPOH1 is an organism that has not been described before. Further characterization will determine the taxonomic status of strain NPOH1.

Feeding cattle supplements of nitrate or nitroethane, a

less toxic analog of nitropropanol, results in enhanced rates of ruminal nitropropanol metabolism (13, 31, 35) suggesting that these compounds select for nitropropanol metabolizing microbes. Our findings that strain NPOH1 utilizes nitrate and nitroethane fit with these observations. But, strain NPOH1 used nitrate preferentially over nitropropanol when grown in medium containing both acceptors and this finding may have practical implications. For instance, if nitrate concentrations in the rumen of animals consuming milkvetch forage are sufficiently high, metabolism of nitropropanol may be precluded. In support of this, results obtained with washed cell suspensions of strain NPOH1 suggest that nitrate is not a good inducer of nitropropanol activity. In contrast, nitropropionic acid was a good inducer of nitropropanol activity, but nitropropanol was not a good inducer of nitropropionic acid activity. Thus, the various acceptors utilized by strain NPOH1 do not necessarily induce metabolic activity for all acceptors. Further research needs to be done on the regulatory mechanisms involved. The information gained could be critical for developing strategies to manipulate detoxification.

Rates of ruminal nitropropanol metabolism were not increased when we fed sheep diets containing nitrate or nitroethane. However, it is possible that nitrate and nitroethane may have been present in the ovine rumen fluid

during our *in vitro* incubations and, in light of the above discussion, the presence of these compounds may have precluded induction of nitropropanol activity. Higher rates of nitropropanol metabolism have also been associated with orchardgrass diets than with alfalfa hay diets (35). We have no explanation why rates of nitropropanol metabolism did not increase when sheep were fed orchardgrass hay.

Strain NPOH1 had a much greater ability to metabolize nitropropanol than microbes previously known to metabolize nitropropanol; however, further research needs to be done to determine the contribution of organisms like strain NPOH1 to the detoxification of nitro-toxins in the rumen.

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**APPENDIX. REDUCTION OF NITROPROPANOL BY RUMINAL MICROBES;
INVOLVEMENT OF LOW POTENTIAL ELECTRON CARRIERS.**

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Abstract

Enhanced metabolism of nitropropanol (NPOH), the toxic principle of many milkvetch forages, by ruminal microbes is sought as a means of protecting ruminants from poisoning. Ruminal microbes are known to metabolize NPOH but the microbes primarily responsible for this metabolism and the biochemical reactions involved are as yet unknown. We previously reported that mixed populations of ruminal microbes reduce NPOH to aminopropanol and that the rate of NPOH reduction was enhanced by ferrous and sulfide ions. We report here that NPOH was reduced in a simple system containing clostridial ferredoxin and either hydrogenase or pyruvate dehydrogenase as the electron donating enzyme. In this system, rates of NPOH reduction were increased when ferrous and sulfide ions were added. However, the hydrogenase/ferredoxin system also reduced nitrite and since nitrite did not inhibit the reduction of NPOH, a hypothesis

that ferrous and sulfide ions act by protecting ferredoxin from nitrite inhibition is questioned. Washed cells and cell-free preparations of mixed ruminal bacteria needed ferrous and sulfide ions or the low potential electron carriers methyl viologen or benzyl viologen in order to reduce NPOH. H_2 served as the electron donor in this reaction. The rate of NPOH reduction by *Megasphaera elsdenii* was stimulated by ferrous and sulfide ions. The rate of NPOH metabolism by strain NPOH1, a ruminal bacterium isolated from populations enriched for enhanced rates nitropropanol metabolism, was unaffected by these ions.

Introduction

Several *Astragalus* species (various milkvetchs) that grow on ranges in western North America contain miserotoxin (6). Poisoning of cattle and sheep by the toxic aglycone of miserotoxin, nitropropanol (NPOH), occurs and recent research has been directed towards enhancing the ruminal metabolism of NPOH as a means of preventing poisonings (6-7).

Recent studies have shown that ruminal microbes are able to detoxify NPOH, reducing the nitro-group to produce aminopropanol (2). We also found that the rate of NPOH reduction was markedly enhanced by the addition of ferrous and sulfide ions. A possible analogous nitro-group reduction, reduction of nitroethanol to aminoethanol by

clostridial ferredoxin, was studied by Angermaier and Simon (3). They proposed that small amounts of nitrite originating from an unstable intermediate were inhibitory to ferredoxin and that this inhibition could be overcome by adding ferrous and sulfide ions to the system.

Our objectives are to gain an understanding of the NPOH reduction system and we report here on experiments conducted to determine if clostridial ferredoxin could reduce NPOH and to test the proposed function of ferrous and sulfide ions. Results from preliminary experiments using mixed and pure preparations of ruminal microbes are also presented.

Materials and Methods

Experiments with *Clostridium pasteurianum*.

C. pasteurianum was grown at 39°C in medium containing (g/l) K_2KPO_4 , 0.23; KH_2PO_4 , 0.23; $(NH_4)_2SO_4$, 0.45; NaCl, 0.45; $MgSO_4 \cdot 7H_2O$; $CaCl_2$, 0.02; glucose, 6; cellobiose, 4; yeast extract, 20; and resazurin, 0.001, and cysteine-HCl, 1. Prior to inoculation, the medium was adjusted to pH 6.8, autoclaved and 600 ml of a presterilized 8% Na_2CO_3 solution was added while cooling to 39°C under a CO_2 gas phase. Cells harvested using a Sharples continuous flow centrifuge were washed and resuspended using 50 mM Tris-HCl (pH 8.0) plus 1 mM dithiothreitol. Hydrogenase was partially purified from *C. pasteurianum* extracts using DEAE anion exchange

chromotography (5). A unit (U) of hydrogenase is the amount required for the uptake of 1 μmol H_2 /min as measured by reduction of methyl viologen (3). The flow through fractions eluted from the DEAE column were pooled and used as the source of pyruvate dehydrogenase. Ferredoxin from *C. pasteurianum* was purchased from Sigma (St. Louis, MO).

Experiments with ruminal microbes. Mixed ruminal microbes were harvested from 6 l of ruminal contents collected from a fistulated cow which was receiving an alfalfa:corn (9:1) diet. The ruminal contents were squeezed through 2 layers of cheesecloth into prewarmed (39°C) insulated containers and returned to the laboratory for immediate processing. The strained fluid was centrifuged 1X at 150 x g for 10 min to remove protozoa and undigested particulate material and the supernatant was centrifuged at 18,000 x g for 20 min to collect the bacteria. Cells were washed 1X and resuspended using 200 mM Tris-HCl (pH 8.6) or 50 mM Tris-HCl (pH 7.5), each containing 1 mM dithiothreitol.

M. elsdenii B159 was obtained from M. P. Bryant and was available in our culture collection; strain NPOH1 was isolated from mixed ruminal populations enriched for enhanced rates of NPOH metabolism (1). *M. elsdenii* was grown in a 12 l fermentor containing YEL medium (1), strain NPOH1 was grown in side arm flasks containing 400 ml of medium B (1) supplemented with 16 mM formate and 9 mM NPOH with an $\text{H}_2:\text{CO}_2$

(1:1) gas phase. Both organisms were grown at 39°C and cells harvested by centrifugation were washed and resuspended using anaerobic dilution solution (4).

Cell-free preparations. Cell-free extracts were obtained by passing whole cell suspensions through a French pressure cell (20,000 lb²). Intact cells and cell debris were removed from the initial lysates by centrifugation (6,200 x g, 10 min).

Assay procedure. Anaerobic procedures were used throughout the preparation and assay of all suspensions. Assays were started upon the addition of whole cell or enzyme preparations to reaction mixtures contained in 13 x 100 mm tubes. The tubes were closed with rubber stoppers and incubated with continuous agitation at 39°C. Samples were withdrawn at intervals and frozen until analysis for NPOH or nitrite. Values reported are the mean \pm SD from duplicates.

Measurements of NPOH and nitrite. NPOH was measured spectrophotometrically after diazo coupling to para-nitroaniline (2). Nitrite was measured by the colorimetric method of Schneider and Yeary (9).

Results and Discussion

In a previous report (2), unwashed suspensions of ruminal microbes reduced NPOH and the rate of NPOH reduction was stimulated \geq three-fold when ferrous and sulfide ions

were included in the reactions. In the present experiment, washed whole cell preparations and cell-free extracts of mixed ruminal microbes also reduced NPOH (Table 1), but this activity was dependent on the addition of ferrous and sulfide ions or the low potential electron donors methyl viologen or

Table 1. Activities of whole cell and cell-free preparations of mixed ruminal microbes.

Additions to assay mixture	Specific activity ($\mu\text{mol NPOH/mg protein per h}$)	
	Whole cells ^a	Cell-free extracts ^b
No additions	NA ^c	NA
Fe ²⁺ and S ²⁻	0.73 \pm 0.01	11.20 \pm 0.88
Methyl viologen (MV)	ND ^d	27.67 \pm 1.18
Benzyl viologen (BV)	ND	18.48 \pm 0.83
Fe ²⁺ , S ²⁻ and MV	ND	33.61 \pm 0.10
Fe ²⁺ , S ²⁻ and BV	ND	23.09 \pm 2.52

^aWhole cells (5.9 mg protein) were incubated (8 h) under 100% H₂ in 1.5 ml 200 mM Tris-HCl (pH 8.6) containing 1 mM dithiothreitol, 10 mM nitropropanol and where indicated FeSO₄ and Na₂S (each at 2.5 mM).

^bCell-free extracts (0.2 mg protein) were incubated (4 h) under 100% H₂ in 1.5 ml 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 10 mM nitropropanol and where indicated, 3 mM FeSO₄, 3 mM Na₂S, 10 mM methyl viologen or 10 mM benzyl viologen.

^cNA; no activity.

^dND; not done.

benzyl viologen. H_2 served as the reductant in the reduction of NPOH. The specific NPOH activity of mixed ruminal preparations varied considerably between extracts prepared from cells harvested on different days and the values shown in Table 1 are from a preparation with high activity. It is possible that endogenous inhibitors were present in the cell-free extracts since the specific NPOH activity decreased proportionally ($R^2=0.96$) as amounts of extracts were increased in the reaction mixtures containing the ferrous and sulfide ions. However, specific NPOH activity remained relatively constant in assay mixtures containing methyl viologen and increasing amounts of extract (not shown).

Strain NPOH1, a ruminal bacterium isolated from populations enriched for nitropropanol metabolism, has a much greater capacity to metabolize nitropropanol than ruminal microbes previously known to possess this ability. The rate of NPOH metabolism of nitropropanol metabolism by strain NPOH1 was affected little by ferrous and sulfide ions; however, rates of NPOH metabolism by *M. elsdenii* and *C. pasteurianum* were increased greater than two-fold (Table 2).

Washed cell suspensions of *C. pasteurianum* and purified ferredoxin, reduced either by hydrogenase or pyruvate dehydrogenase, reduced nitropropanol (Table 3). Reduction of NPOH by the preparations containing ferredoxin was proportional to the amount of ferredoxin and the amount

Table 2. Affects of ferrous and sulfide ions on nitropropanol metabolism by ruminal microbes.

Organism ^a	Specific activity (μ mol NPOH/mg protein per h)	
	Without Fe and S	With Fe and S
<i>M. elsdenii</i>	0.06 \pm <.01	0.21 \pm <.01
<i>C. pasteurianum</i>	0.03 \pm <.01	0.08 \pm <.01
Strain NPOH1	0.59 \pm 0.05	0.57 \pm <.01

^aWhole cell suspensions were incubated 8 h in 1.5 ml anaerobic dilution solution containing 5 mM nitropropanol with or without additions of FeSO₄ and Na₂S (each at 5 mM). Incubations with *M. elsdenii* and *C. pasteurianum* contained 8.0 mg cell protein, incubations with strain NPOH1 contained 0.9 mg cell protein.

of enzyme in the assay mixture. Ferrous and sulfide ions stimulated the reduction of NPOH by these preparations, although not to the same magnitude as that observed with preparations of mixed ruminal microbes. Ferredoxin and other low potential e⁻ carriers are distributed widely among anaerobic bacteria but whether or not these carriers contribute nonspecifically to the reduction of NPOH within the rumen remains to be determined.

Angermaier and Simon (3) proposed that ferrous and sulfide ions enhanced the ferredoxin mediated reduction of nitroethanol by protecting ferredoxin from inhibition by nitrite. Our experiments with hydrogenase/ferredoxin did not support this explanation as NPOH reduction was not appreciably

Table 3. Activities of whole cell suspensions and partially purified enzyme preparations of *C. pasteurianum*.

	Specific activity ($\mu\text{mol NPOH/mg protein per h}$)		
	Whole cells ^a	Hydrogenase and ferredoxin ^b	Clastic and ferredoxin ^c
No Fe^{2+} & S^{2-}	0.07 ± 0.09	0.11 ± 0.03	$0.07 \pm <.01$
With Fe^{2+} & S^{2-}	0.14 ± 0.01	0.23 ± 0.01	$0.12 \pm <.01$

^aWhole cells (44 mg protein) were incubated 8 h in 3.0 ml 50 mM Tris-HCl (pH 8.0) containing 10 mM nitropropanol with or without 2.5 mM FeSO_4 and 2.5 mM Na_2S .

^bHydrogenase (6.2 mg protein; 0.008 U activity) and 48 μg ferredoxin were incubated 8 h in 1.2 ml 50 mM Tris-HCl (pH 8.0) containing 10 mM nitropropanol with or without 0.4 mM FeSO_4 and 0.4 mM Na_2S .

^cPyruvate dehydrogenase (7.8 mg protein) and 36 μg ferredoxin were incubated 7 h at 39°C in 1 ml 30 mM phosphate buffer (pH 6.5) containing 10 mM nitropropanol, 50 μg CoA, and 4 mM pyruvate with or without 0.4 mM FeSO_4 and 0.04 mM Na_2S .

inhibited by nitrite, either in the presence or absence of ferrous and sulfide ions (Figure 1A). The amount of nitrite added to our system was well above that anticipated to be inhibitory to ferredoxin (<0.4 mM). Nitrite was rapidly reduced by the hydrogenase/ferredoxin system when ferrous and sulfide ions were absent but not when these ions were included in the reaction mixture (Figure 2A). The reduction of nitrite by ferredoxin has been reported by others (8). Rates of NPOH metabolism were affected little by the ferrous

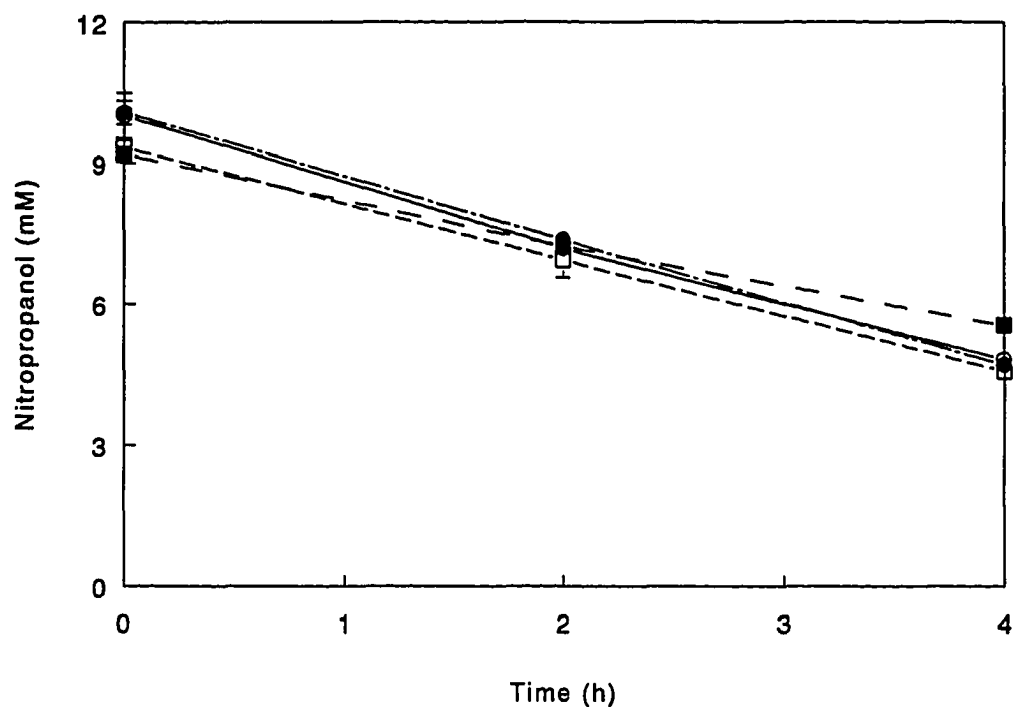


FIGURE 1A. Reduction of nitropropanol by clostridial ferredoxin/hydrogenase. Reaction mixtures contained in 0.6 ml 50 mM Tris-HCl (pH 8.0); 0.8 mM dithiothreitol, 10 mM nitropropanol, 300 μ g ferredoxin, 2.6 mg (0.008 U activity) hydrogenase and 0 (circles) or 20 mM (squares) sodium nitrite without (open symbols) or with (closed symbols) FeSO_4 and Na_2S (each at 0.4 mM). Mixtures were incubated under 100% H_2 .

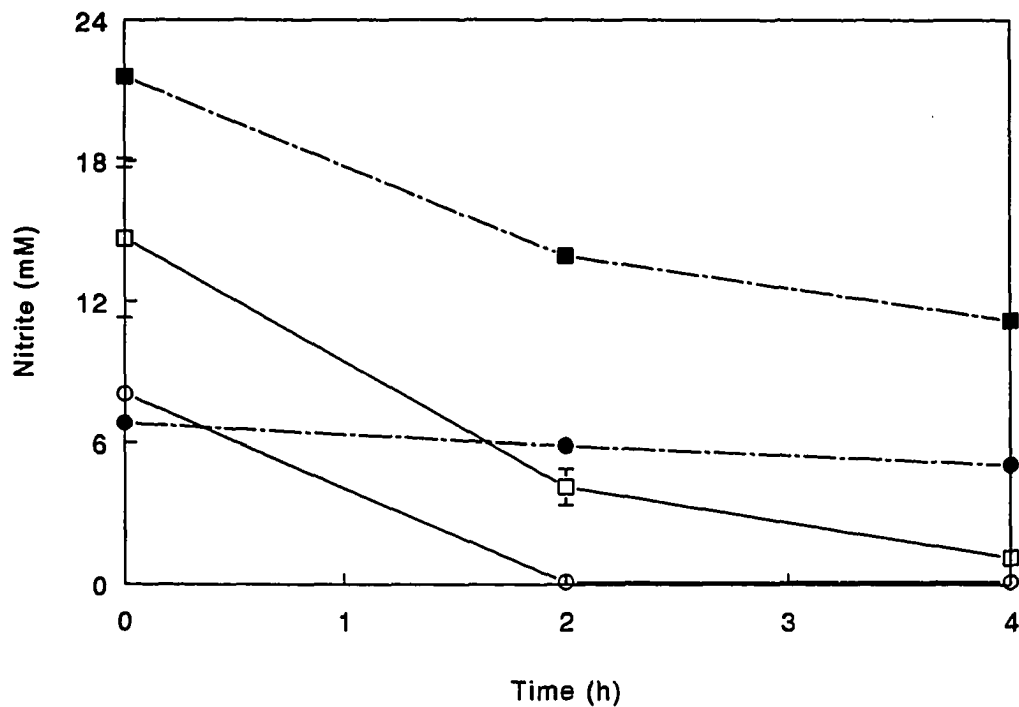


Figure 2A. Reduction of nitrite by clostridial ferredoxin/hydrogenase. Reaction mixtures contained in 0.6 ml 50 mM Tris-HCl (pH 8.0); 0.8 mM dithiothreitol, 10 mM nitropropanol, 300 μ g ferredoxin, 2.6 mg (0.008 U activity) hydrogenase and 10 mM (circles) or 20 mM (squares) sodium nitrite without (open symbols) or with (closed symbols) FeSO₄ and Na₂S (each at 0.4 mM). Mixtures were incubated under 100% H₂.

and sulfide ions in this experiment; however, this may have been because of the larger amount of ferredoxin added to the assay mixtures.

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